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<b>(54) Title:</b> AN ENZYME EXHIBITING CELLULASE ACTIVITY  <b>(57) Abstract</b>  An enzyme which exhibits cellulase activity, which enzyme is producible by a strain of <i>Bacillus</i> spp., NCIMB 40250, or a related <i>Bacillus</i> spp. strain, or a derivative of said cellulase. In particular, the enzyme is an endoglucanase with an apparent molecular weight of 75, 56 or 45 kD or a cleavage product thereof with endoglucanase activity. An enzyme which comprises a core region derived from an endoglucanase combined with a cellulose-binding domain derived from another cellulase enzyme, or which comprises a core region derived from another cellulase enzyme combined with a cellulose-binding domain derived from an endoglucanase.		

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## AN ENZYME EXHIBITING CELLULASE ACTIVITY

## FIELD OF THE INVENTION

- 5 The present invention relates to an enzyme exhibiting cellulase activity, a DNA construct encoding the enzyme, a cellulolytic agent comprising the enzyme and a detergent composition containing the enzyme.

## 10 BACKGROUND OF THE INVENTION

Biomass which largely consists of cellulose, hemicellulose and lignin has attracted increasing attention as an important renewable source of energy (including nutritional energy). The amount of carbon fixed by photosynthesis has been estimated to be  $100 \times 10^9$  tons per year worldwide, and half of that is contained in cellulose. If this material, or at least a significant part of it, could be converted into liquid fuel, gas and feed protein, this would constitute a significant contribution to solving the problem of recycling and conservation of resources. However, it has been found difficult to develop an economically viable process of converting cellulosic material into fermentable sugars.

- 25 The currently most promising of the suggested processes involves the use of enzymes which are able to degrade cellulose. These enzymes which are collectively known as cellulases are produced by a number of microorganisms, including fungi (e.g. Trichoderma reesei, Humicola insolens, Fusarium oxysporum, etc.) and bacteria (e.g. Clostridium thermocellum, Cellulomonas spp., Thermonospora spp., Bacterioides spp., Microbispora bispora, etc.). The economics of the production of fermentable sugars from biomass by means of such enzymes is not yet competitive with, for instance, the production of glucose from starch by means of  $\alpha$ -amylase due to the inefficiency of the cellulase enzymes. The most significant problems connected with the use of cellulases is their low specific activity and the high cost of

their production. Therefore, there is a need to develop cellulases which are more efficient in degrading cellulosic materials into fermentable sugars.

5 Apart from their utility for the degradation of biomass, cellulases have also been suggested for use in detergent compositions for the treatment of cotton-containing fabrics which largely consist of cellulose. It is well known that repeated washing of cotton-containing fabrics generally causes  
10 a pronounced, unpleasant harshness in the fabric due to the presence of amorphous regions in the cellulose fibres, which regions form protruding parts on the otherwise smooth fibres. Several methods for overcoming this problem have previously been suggested. For example, US 1.368.599 of Unilever Ltd. teaches  
15 the use of cellulases for reducing the harshness of cotton-containing fabrics. Also, US 4.435.307 (of Novo Industri A/S) teaches the use of a cellulytic enzyme derived from Humicola insolens as well as a fraction thereof as a harshness reducing detergent additive. Other uses of cellulases mentioned in the  
20 art include soil removal from and colour clarification of fabric (cf. for instance EP 220 016).

Although the use of cellulase enzymes for harshness reduction of cotton-containing fabrics was suggested and demonstrated nearly  
25 20 years ago the mechanism of this process has not been elucidated and is still not known in detail. Among other things, this is due to the multiplicity of the enzymes and the enzyme-catalyzed reactions involved. As a matter of fact, cellulases generated in nature e.g. by microbial species are indeed complex  
30 mixtures of cellulases. Accordingly, the conversion of naturally occurring materials, like cotton, catalyzed by cellulases is exceedingly difficult to analyze in detail.

Due to these circumstances, the practical exploitation of  
35 cellulases for harshness reduction and prevention as well as colour clarification, however desirable, has not become widespread and of great practical utility: it is difficult to



optimise production of multiple enzyme systems and thus to implement industrial cost-effective production of cellulase enzymes, and their actual use has been hampered by difficulties arising from the need to employ rather large quantities of the cellulases to achieve the desired reduction and prevention of the harshness of cotton fabrics: for instance, addition of large quantities of the enzymes to detergent compositions is not compatible with the optimal function of other ingredients in the detergent formulation nor is the addition of very large quantities of enzymes to the detergent composition in the interests of, e.g., consumer safety.

The object of the present invention is therefore to provide cellulase enzymes with a high specific activity.

#### SUMMARY OF THE INVENTION

The present invention relates to an enzyme which exhibits cellulase activity, which enzyme is producible by a strain of Bacillus spp., NCIMB 40250, or by a related Bacillus spp. strain, or a derivative of said cellulase. The strain NCIMB 40250 was deposited on 18 January, 1990, in the National Collection of Industrial and Marine Bacteria, 23 St Machar Drive, Aberdeen, Scotland, UK, in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure.

In the present context, the expression "enzyme exhibiting cellulase activity" is meant to be understood as an enzyme which is involved in the process of cellulose degradation. There are three different types of cellulases which act synergistically to produce soluble sugars: endoglucanases which show affinity for cellulose and which attack amorphous regions of low crystallinity in the cellulose fibre resulting in the formation of free ends; exoglucanases which initiate degradation from the non-reducing chain ends by removing cellobiose units; and  $\beta$ -glucosidases which hydrolyse cellobiose to glucose.

- The expression "related Bacillus spp. strain" is intended to indicate a strain belonging to the same Bacillus species as the strain NCIMB 40250 or a strain of a closely related species. The species to which the strain NCIMB 40250 belongs has been identified as Bacillus lautus. The scope of the present invention is also intended at least to include cellulase enzymes producible by other Bacillus lautus strains than NCIMB 40250.
- 10 The term "derivative" is intended to indicate a protein which is derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end of the the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid
- 15 sequence, deletion of one or more amino acids at either or both ends of the native protein or at one or more sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence.
- 20 Although the enzyme of the invention may be produced by cultivating the Bacillus spp. strain NCIMB 40250 or a related strain and isolating the enzyme from the culture, it will generally be more advantageous to produce the enzyme by recombinant DNA techniques which make it possible to optimize
- 25 the yield of the enzyme produced. Furthermore, cloned genes encoding the enzymes may be modified in order to provide enzymes with improved properties.
- Thus, in another aspect, the present invention relates to a DNA
- 30 construct which comprises a DNA sequence encoding an enzyme exhibiting cellulase activity, which enzyme is derivable from a strain of Bacillus spp., NCIMB 40250, or a related Bacillus spp. strain, or a derivative of said cellulase. The invention further relates to an expression vector which carries an inserted DNA
- 35 construct as indicated above, as well as to a cell transformed with the DNA construct or with the vector.

In a still further aspect, the invention relates to a cellulolytic agent capable of degrading amorphous regions of cellulose fibres, the agent comprising an enzyme exhibiting cellulase activity as defined above.

5

The invention also relates to a detergent composition comprising the cellulolytic agent. The cellulase enzyme of the invention has surprisingly been found to be more stable during washing (for 60 minutes at 40°C) in the presence of conventional  
10 detergents than a commercial cellulase preparation (Celluzyme™, a cellulase preparation isolated from Humicola insolens, available from Novo Nordisk, A/S). The cause of the increased stability may reside in the alkalophilic nature of the enzyme (see example 5 below). It is further speculated that it may also  
15 be ascribed to stability towards oxidation or towards the proteases commonly included in detergents. If so, the cellulase enzyme of the invention may also show increased storage stability in liquid detergents containing proteases.

## 20 DETAILED DISCLOSURE OF THE INVENTION

The cellulase enzyme of the present invention is preferably one which exhibits endoglucanase activity (referred to in the following as an endoglucanase), in particular one which exhibits  
25 an endoglucanase activity of at least about 10, more preferably at least about 20, most preferably at least about 25, such as about 30, CMC-endoase units per mg of total protein under alkaline conditions. The endoglucanase activity is determined as the viscosity decrease of a solution of carboxymethyl cellulose  
30 (CMC) after incubation with the enzyme of the present invention under the following conditions:

A substrate solution is prepared, containing 35 g/l CMC (Hercules 7 LFD) in 0.1 M tris buffer at pH 9.0. The enzyme sample  
35 to be analyzed is dissolved in the same buffer.

10 ml substrate solution and 0.5 ml enzyme solution are mixed

and transferred to a viscosimeter (e.g. Haake VT 181, NV sensor, 181 rpm), thermostated at 40°C.

5 Viscosity readings are taken as soon as possible after mixing and again 30 minutes later. The amount of enzyme that reduces the viscosity by one half under these conditions is defined as 1 CMC-endoase unit.

10 It should be noted that the endoglucanase of the invention is one which is active (in terms of CMC-endoase activity) under alkaline conditions. More specifically, the endoglucanase is one which has a pH optimum at a pH of about 7.5-10.5. Contrary to several known cellulases which are active at an acid pH and relatively inactive at alkaline pH values, this characteristic 15 makes the endoglucanase of the invention particularly useful for washing purposes, in particular as an ingredient of a detergent composition, as washing of clothes is typically conducted under alkaline conditions due to the alkalinity of most washing detergents. Alkalophilic cellulases are known, e.g. from EP 271 20 004, but they are not indicated to have a high affinity for cellulose, which is the case with the cellulase enzyme of the present invention which also exhibits a higher specific activity.

25 The enzyme of the present invention is preferably one which is active at the temperatures at which clothes are typically washed, which is usually a temperature of up to about 60°C. Thus, the native enzyme isolated from strain NCIMB 40250 is active at temperatures between about 45 and 65°C. This, however, 30 does not preclude the possibility that the enzyme may, under certain conditions, be active at temperatures above 65°C.

One enzyme according to the invention is an endoglucanase with an apparent molecular weight of 75 kD or a cleavage product 35 thereof exhibiting endoglucanase activity. The term "cleavage product" is intended to indicate a shorter form of the enzyme resulting from, for instance, chemical or enzymatic cleavage

(e.g. by means of a suitable protease) after recovery of the enzyme or from posttranslational processing by the organism producing the enzyme, e.g. N- and/or C-terminal processing, which may give rise to a mature form of the enzyme. A specific  
5 example of a cleavage product of the ~75 kD enzyme which is of interest for the present purpose is a product of approximately 58 kD produced on cultivating a host organism transformed with DNA encoding the ~75 kD enzyme. The ~75 kD enzyme (and its ~58  
10 kD cleavage product) are referred to in the following examples as Endo1.

The enzyme of the invention may be an endoglucanase encoded by the DNA sequence shown in the appended Sequence Listing ID#1 (showing the sequence of the ~75 kD enzyme), or a modification  
15 thereof encoding a derivative of said endoglucanase.

Endoglucanase derivatives may conveniently be provided by suitably modifying the DNA sequence coding for the native endoglucanase. Examples of suitable modifications of the DNA  
20 sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the endoglucanase, but which may correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and  
25 therefore, possibly, a different polypeptide structure without, however, impairing the properties of the endoglucanase. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence and deletion of one or  
30 more nucleotides at either end of or within the sequence. Such modifications of DNA coding for native proteins are well known and widely practised in the art.

Another enzyme according to the invention is an endoglucanase  
35 with an apparent molecular weight of 56 kD or a cleavage product thereof (as defined above) exhibiting endoglucanase activity. In example 2 below, this enzyme is referred to as Endo2.

The enzyme of the invention may be an endoglucanase encoded by the DNA sequence shown in the appended Sequence Listing ID#3 (showing the sequence of the ~56 kD enzyme), or a modification thereof (as defined above) encoding a derivative (as defined above) of said endoglucanase.

A further enzyme according to the invention is an endoglucanase with an apparent molecular weight of 45 kD or a cleavage product thereof (as defined above) exhibiting endoglucanase activity. A specific example of such a cleavage product is a protein of approximately 30 kD produced on cultivating a host organism transformed with DNA encoding the ~45 kD enzyme. In example 3 below, the ~45 kD enzyme is referred to as Endo3A.

15

The enzyme of the invention may be an endoglucanase encoded by the DNA sequence shown in the appended Sequence Listing ID#5 (showing the DNA sequence encoding the ~45 kD product), or a modification thereof (as defined above) encoding a derivative (as defined above) of said endoglucanase.

Other enzymes exhibiting endoglucanase activity produced from endoglucanase clone 3 (cf. example 3 below) are proteins of approximately 60 and 56 kD, referred to as Endo3B and Endo3C, respectively.

A still further enzyme according to the invention is an endoglucanase with an apparent molecular weight of 92 kD or a cleavage product thereof (as defined above) exhibiting endoglucanase activity. In example 4 below, this enzyme is referred to as Endo4. Other enzymes exhibiting endoglucanase activity produced from endoglucanase clone 4 (cf. example 4 below) are proteins of approximately 74 and 71 kD. Either or both of these may be individual enzymes or cleavage products of the ~92 kD enzyme.

- It has been found that enzymes of the invention, e.g. Endo1 and Endo3A, are composed of a core region comprising the catalytically active domain and a region comprising a domain whose function is to mediate binding to cellulosic substrates
- 5 (i.e. the cellulose-binding domain; this corresponds to a similar domain in an endocellulase from Bacillus subtilis (Nakamura et al., 1987). For example, the full-length ~75 kD form of Endo1 comprises a core region and a C-terminal cellulose-binding domain which, in some cases, may be cleaved
- 10 off proteolytically leaving a core region of ~58 kD. The presence of the cellulose-binding domain has been found to be important for obtaining a colour clarification effect in prewashed textiles (cf. example 6 below).
- 15 Based on this finding, it may be possible to generate novel derivatives of cellulase enzymes which, for instance, combine a core region derived from an endoglucanase of the present invention with a cellulose-binding domain derived from another cellulase enzyme (e.g. one derived from a Cellulomonas fimi
- 20 cellulase). Alternatively, it may be possible to combine a core region derived from another cellulase enzyme with a cellulose-binding domain derived from an endoglucanase of the present invention. In a particular embodiment, the core region may be derived from a cellulase enzyme which does not, in nature,
- 25 comprise a cellulose-binding domain, and which is C-terminally extended with a cellulose-binding domain derived from an endoglucanase of the present invention. In this way, it may be possible to construct cellulase enzymes with improved binding properties.
- 30
- The DNA construct of the invention may be one which comprises a DNA sequence encoding any one of the enzymes described above, or derivatives of these enzymes as defined above. The DNA construct may be of genomic or cDNA origin, for instance obtained by
- 35 preparing a genomic or cDNA library of an appropriate Bacillus spp. strain (e.g. strain NCIMB 40250 or a related strain) and screening for DNA sequences coding for all or part of the

appropriate cellulase by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Maniatis et al., 1982).

- 5 The expression vector of the invention carrying the inserted DNA construct encoding the enzyme of the invention may be any vector which is capable of replicating autonomously in a given host organism, typically a plasmid or bacteriophage. In the vector, the DNA sequence encoding the enzyme of the invention should be  
10 operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell and may be derived from genes encoding proteins either homologous or heterologous to the host organism. The promoter is preferably derived from a gene encoding a protein  
15 homologous to the host organism. Examples of suitable promoters are lac of E.coli, dagA of Streptomyces coelicolor and amyL of Bacillus licheniformis.

- The expression vector of the invention further comprises a DNA  
20 sequence enabling the vector to replicate in the host cell. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110 and pIJ702.

- The expression vector may further comprise a DNA sequence coding  
25 for a signal peptide in order to provide extracellular expression of the enzyme. The DNA sequence may, for instance, be the signal sequence from the  $\alpha$ -amylase gene of B. licheniformis.

- The vector may also comprise a selectable marker, e.g. a gene  
30 whose product confers antibiotic resistance, such as ampicillin, chloramphenicol or tetracycline resistance, or the dal genes from B.subtilis or B.licheniformis.

- The procedures used to ligate the DNA sequences coding for the  
35 enzyme of the invention and the promoter, respectively, and to insert them into suitable vectors containing the information necessary for replication in the host cell, are well known to



persons skilled in the art (cf., for instance, Maniatis et al., op.cit.).

5 The host cell of the present invention may be transformed with the DNA construct of the invention encoding the cellulase enzyme described above. In this case, the DNA construct may conveniently be integrated in the host chromosome which may be an advantage as the DNA sequence coding for the cellulase is more likely to be stably maintained in the cell. Integration of  
10 the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous recombination.

15 Alternatively, the host cell may be transformed with an expression vector as described above.

The host cell used in the process of the invention may be any suitable bacterium which, on cultivation, produces large amounts of the enzyme of the invention. Examples of suitable bacteria  
20 may be grampositive bacteria such as bacteria belonging to the genus Bacillus, e.g. Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans or Bacillus lautus, or  
25 gramnegative bacteria such as Escherichia coli. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known per se.

30 In a yet further aspect, the present invention relates to a method of producing a cellulase enzyme of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the cellulase or derivative thereof and recovering the cellulase or derivative  
35 thereof from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional

medium suitable for growing bacteria. The cellulase may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, if necessary after disruption of the cells, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

10

In a particular embodiment of the method of the invention, the cellulase is recovered in mature form, either as a result of posttranslational processing of a proenzyme as explained above or as a result of appropriate modifications of the DNA sequence encoding the enzyme in the form of deletions of DNA corresponding to truncations in the N- and/or C-terminal sequences of the enzyme.

There is reason to believe that different cellulases may exert a synergistic effect with respect to the degradation of cellulose. The cellulolytic agent of the invention may therefore advantageously comprise a combination of two or more cellulase enzymes of the invention or a combination of one or more cellulase enzymes of the invention with one or more other enzymes exhibiting cellulase activity. Such cellulases may be endocellulases or exocellulases dependent on the intended use of the cellulolytic agent (e.g. the degree of cellulose degradation aimed at). The other cellulases may be selected from those which may be isolated from species of Hemicola such as Hemicola insolens (e.g. strain DSM 1800), Fusarium such as Fusarium oxysporum (e.g. strain DSM 2672), Myceliophthora such as Myceliophthora thermophila, Erwinia such as Erwinia chrysanthemum (cf. M.H. Boyer et al., Eur. J. Biochem. 162, 1987, pp. 311-316), Trichoderma such as Trichoderma reesei, Microbispora such as Microbispora bispora, Neocallimastix such as Neocallimastix frontalis, Piromonas such as Piromonas communis, Robillarda spp., Cellulomonas such as Cellulomonas

fimi, Clostridium such as Clostridium thermocellum, Pseudomonas spp., Thermonospora spp., Bacterioides spp. or Ruminococcus spp.

The cellulolytic agent of the invention may suitably be in the form of a non-dusting granulate, stabilized liquid or protected enzyme. Non-dusting granulates may be produced e.g. according to US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The cellulolytic agent may suitably exhibit an endoglucanase activity of 500-10,000 CMC-endoase units (as defined above) per gram of the agent. The cellulolytic agent is suitably a detergent additive which may comprise one or more other enzymes, such as a protease, lipase and/or amylase, conventionally included in detergent additives.

The detergent composition of the invention comprising the cellulolytic agent described above additionally comprises one or more surfactants which may be of the anionic, non-ionic, cationic, amphoteric, or zwitterionic type as well as mixtures of these surfactant classes. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS), alpha olefin sulfonates (AOS), alcohol ethoxy sulfates (AES) and alkali metal salts of natural fatty acids.

The detergent composition of the invention may contain other detergent ingredients known in the art as e.g. builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, enzyme stabilizers, etc.

The detergent composition of the invention may be formulated in

- any convenient form, e.g. as a powder or liquid. The enzyme may, if required, be stabilized in a liquid detergent by inclusion of enzyme stabilizers as indicated above. Usually, the pH of a solution of the detergent composition of the invention will be
- 5 7-12 and in some instances 7.0-10.5. Other detergent enzymes such as proteases, lipases or amylases may be included in the detergent composition of the invention, either separately or in a combined additive as described above.
- 10 The softening, soil removal and colour clarification effects obtainable by means of the cellulase enzyme of the invention generally require a concentration of the cellulase in the washing solution corresponding to an endoglucanase activity of
- 15 5 - 200 CMC-endoase units per liter. The detergent composition of the invention is typically employed in concentrations of 0.5 - 20 g/l in the washing solution. Consequently, the cellulase concentration of the detergent composition of the invention is about 0.3 - 400 CMC-endoase units per gram. In general, it is most convenient to add the detergent additive in amounts of 0.1
- 20 - 5 % w/w or, preferably, in amounts of 0.2 - 2 % of the detergent composition. For special applications, however, for instance when the detergent composition is to be used for colour clarification or harshness reduction of fabric which has been damaged by repeated washing, it may be convenient to include a
- 25 much larger amount of the cellulolytic agent, such as about 20% w/w.

- In a still further aspect, the present invention relates to a method of reducing the rate at which cellulose-containing
- 30 fabrics become harsh or of reducing the harshness of cellulose-containing fabrics, the method comprising treating a cellulose-containing fabric with a cellulolytic agent as described above. The method of the invention may be carried out by treating cellulose-containing fabrics during washing. The cellulolytic
- 35 agent may either be added as such in the amount required to obtain the desired effect, or it may be added as an ingredient of a detergent composition as described above. However, if

desired, treatment of the fabrics may also be carried out during soaking or rinsing or simply by adding the cellulolytic agent to water in which the fabrics are or will be immersed.

- 5 The cellulolytic agent of the invention may also be employed to obtain colour clarification of cellulose-containing fabrics. After repeated washing, such fabrics often develop a grayish appearance. This effect is particularly evident with coloured fabrics, especially dark fabrics, and may probably be ascribed  
10 to undyed parts of the cellulose fibres becoming apparent when the cellulose fibres of which the fabric is composed are damaged by mechanical forces. The damaged parts of the fibres are assumed to be more amorphous than intact cellulose fibres and therefore more susceptible to the action of the cellulases of  
15 the present invention. The colour clarification effect is more pronounced when the cellulolytic agent contains an endoglucanase which comprises a cellulose-binding domain (cf. example 6 below).
- 20 Accordingly, the present invention further relates to a method of treating a coloured, cellulose-containing fabric in order to provide colour clarification, the method comprising treating the cellulose-containing fabric with a cellulolytic agent according to the invention. The method of the invention may be carried out  
25 by treating cellulose-containing fabrics in an aqueous medium during washing. The cellulolytic agent may either be added as such in the amount required to obtain the desired effect, or it may be added as an ingredient of a detergent composition as described above. However, if desired, treatment of the fabrics  
30 may also be carried out during soaking or rinsing or simply by adding the cellulolytic agent to water in which the fabrics are or will be immersed. For colour clarification purposes, the aqueous medium may suitably exhibit an endoglucanase activity of more than about 250 CMC-endoase units per liter of the aqueous  
35 medium.

It may furthermore be possible to employ a cellulolytic agent

according to the invention to provide a localized variation in the colour of a fabric to impart a "stone-washed" appearance to the fabrics (for the use generally of cellulase enzymes for this purpose, see for instance EP 307 564).




5

The cellulolytic agent of the invention is also contemplated to be useful in the field of paper pulp processing, e.g. pulp drainage (for the use generally of cellulase enzymes for this purpose, see for instance EP 262 040), as well as for deinking  
10 of paper intended for recycling (for the use generally of cellulase enzymes for this purpose, see for instance JP 59-9299 or JP 63-59494).

The invention is further illustrated by the following examples  
15 which are not in any way intended to limit the scope of the invention, with reference to the appended drawings, wherein

Fig. 1    Restriction maps of the *Bacillus* spp. DNA insert  
          contained on the plasmids in the 4 endoglucanase-  
20           positive *E.coli* clones. The position and direction of  
          transcription of endoglucanase 1 (Endo1, pPL517) and  
          endoglucanase 2 (Endo2, pPL382) is indicated. Restriction  
          enzyme sites are indicated as follows: PstI (P),  
          HindIII (H), SmaI, (S), SalI (Sa), BamHI (Ba), BglII  
25           (B), SphI (Sp), EcoRI (E). ■ indicates pBR322 DNA.

Fig. 2    Restriction maps of different plasmids carrying the  
          Endo1 gene (□). The  $\beta$ -lactamase promoter of pBR322  
          is indicated by the arrow (Pb) and the direction of the  
30           transcription of the Endo1 gene is indicated by the  
          arrow (Pb). (■): pBR322, (■): "tail" of pBR322  
          encoded amino acids. Restriction enzyme sites are  
          indicated as follows: PstI (P), BglII (B), EcoRI, (E),  
          ClaI (C). The endoglucanase activity in extracts of  
35           E.coli MC1000 containing the indicated plasmids is  
          shown to the right as (A) cellulase units/ml culture  
          medium.

- Fig. 3 Effect of temperature on the cellulase (Endo1) in extracts of E.coli MC1000 (pPL517) and on the multiple cellulase activities in the supernatant of Bacillus spp. PL236. The activity was measured at the temperature indicated after an incubation period of 30 min. See Materials and Methods. The activity at the different temperatures is presented as cellulase units/ml of the original culture volume.
- Fig. 4 Relevant restriction sites and sequencing strategy of the endoglucanase Endo1 indicated as endocellulase 1 in the figure. (  ): extent and direction of sequence reactions. Abbreviations: EcoRI (E), PstI (P), BglII (Bg), BstNI (Bs), AvaI (A), ClaI (C), BamHI (Ba).
- Fig. 5 is a restriction map of plasmid pDN 2801. Restriction enzyme sites are indicated as follows: EcoRI (E), BglII (Bg), HindIII (H), SmaI (Sm), SalI (Sa), SphI (Sp), PstI (P), EagI (Ea), ClaI (C), BamHI (B). CAT indicates the gene mediating chloramphenicol resistance. P<sub>m</sub> indicates the Bacillus maltogenic  $\alpha$ -amylase promoter.
- Fig. 6 Restriction maps of different plasmids (B.subtilis replication origin) carrying the Endo1 gene. The maltogenic alpha-amylase promoter is indicated by the arrow (P<sub>m</sub>), which also indicates the direction of the transcription of the Endo1 gene; (  ): pDN2801, (  ): tail of PDN2801 encoded sequences. Restriction enzyme sites are indicated as follows: EagI (Ea), BglII (Bg), PstI (P), BamHI (B). The endocellulase activity in extracts of B.subtilis DN 1815 containing the indicated plasmids is shown to the right (A) as endocellulase units/ml culture medium.

- Fig. 7a Construction of pTL05 and pLA03. (■): represents the  
and b C-terminal "tails" encoded by vector sequences. (↑):  
indicates the expected C-terminal cleavage site.  
Restriction sites are abbreviated as follows: PstI (P),  
5 Pvu (Pv), BamHI (B), BglII (Bg), SalI (S).
- Fig. 8a Construction of pCH57. (▨): Signal sequence of the  
and b alpha-amylase. (▩): signal sequence of the Endo1  
glucanase (■): "direct repeat" e.g. start of the  
10 mature Endo1 gene. (●) ribosome binding site of the  
alpha-amylase. (○) ribosome binding site of the Endo1  
glucanase (➡): alpha-amylase promoter. Restriction  
sites are abbreviated as follows: PstI (P); SalI (S);  
15 KpnI (K); EagI (Ea); BglII (Bg).
- Fig. 9 Relevant restriction sites and sequencing strategy of  
the endoglucanase Endo2 (indicated as endocellulase 2  
in the figure). (→): extent and direction of  
sequence reactions. Abbreviations: ScaI (Sc), SacI  
20 (Sa), XmnI (X), HindII (Hc), HindIII (H), PstI (P).
- Fig. 10 Zymogram showing the molecular weight of the active  
proteins resulting from different plasmids carrying the  
original endoglucanase 3 clone (pPL591) as well as  
25 deletions in the original insert.
- Fig. 11 Restriction maps of the endoglucanase 3 clone (in-  
dicated in the figure as endocellulase 3) (pPL591) and  
plasmids containing deletions in the original insert.  
30 The position of the endoglucanase genes Endo3A, Endo3B  
and Endo3C, on the insert in plasmid pPL591 predicted  
from the data shown in the zymogram (Fig. 10) is shown  
on the restriction map. Restriction enzyme sites are  
indicated as follows: HindIII (H), SmaI (S), EcoRI (E).  
35



EXAMPLESMATERIALS AND METHODS5 a) Bacterial strains and plasmids

The donor strain, Bacillus spp., strain PL236, was isolated from a compost sample from Lyngby, Denmark, on the basis of its high cellulolytic activity. A sample of this strain was deposited on 18th January, 1990 in the National Collection of Industrial and Marine Bacteria, 23 St. Machar Drive, Aberdeen, Scotland, with the accession No. NCIMB 40250.

The following E. coli strains and plasmid were used: MC 1000 (araD139), (ara, leu)7697, lacX74, galU, galK, rpsL) (Casabádan et al., 1980); CSR603 (F-, thr-1, leuB6, proA2, prh-1, RecA1, argE3, thi-1, uvrA6, ara-14, lacY1, galK2, xyl-5, mtl-1, rpsL31, tsx-33, -, supE44) (Sancar et al., 1979); PL248 is MC1000 containing the plasmid pNF2690 which contains the replication origin and the kanamycin resistance gene from pACYC177 (Chang and Cohen, 1978) and the cI857 repressor gene from the coliphage lambda; pBR322 (Bolivar, 1977); pUN121 (Nilsson et al., 1983) pUC18 (Yanisch-Perron et al., 1985); pPLc28 (Remaut et al., 1981); pPL170 (Jørgensen, P.L., 1983);

25 For the experiments with B. subtilis, the following B. subtilis strains and plasmids were used: DN1885 (amyE, amyR, spo<sup>+</sup>, pro<sup>+</sup>) (Diderichsen, Novo Industri A/S) is a derivative of B. subtilis 168; PL1801 is a derivative of DN1885 lacking the two main exoproteases (apr<sup>-</sup>, npr<sup>-</sup>); pDN2801 has the origin of replication from pUB110 (Keggins et al., 1987), the Cat gene of pC194 (Horinouchi and Weisblum, 1982) and the maltogenic alpha-amylase promoter (Pm) from B. stearothermophilus (Diderichsen and Christiansen, 1988) followed by a polylinker; The B. subtilis/E.coli shuttle vector pJKK3-1 is described by Kreft et al. 35 (1983); pPL1759 has the origin and kanamycin resistance gene of

pUB110 and the promotor, ribosome binding site and signal sequence from the alpha-amylase gene from B.licheniformis (Stephens et al., 1984).

5    b) Media

Phosphoric acid swollen cellulose (ASC) was prepared from chromatography cellulose (MN 300, Machery, Nagel) as described by Walseth (1952) with the exception that the cellulose powder  
10    was suspended in acetone before treatment with phosphoric acid. The medium for detection of cellulase activity was prepared as standard m)-medium (Maniatis et al., 1982) containing 0.2% ASC or microcrystalline cellulose (Avicel, Merck).

15    Bacillus spp., B.subtilis and E.coli cells were grown in NY medium (von Meyenburg et al., 1982), LB medium (Maniatis et al., 1982), or BPX medium (100 g/l potato starch, 50 g/l barley flour, 0.1 g/l BAN 5000 SKB, 10 g/l sodium caseinate, 20 g/l soybean meal; 9 g/l Na<sub>2</sub> HPO<sub>4</sub>, 12H<sub>2</sub>O, 0.1 g/l Pluronic).

20

The media were solidified by the addition of agar (20 g/l).

Tetracycline (20µg/ml), or kanamycin (10 µg/ml) were added as required.

25

c) Isolation of DNA

To isolate the chromosomal DNA from Bacillus spp. PL236, cells from 250 ml overnight culture were resuspended in 10 ml (50 mM Tris-HCl, pH 8.0, 100 mM EDTA), and incubated with 25 mg  
30    lysozyme for 20 min. at 37°C. To the mixture was added 2 ml of 10% (w/v) SDS, mixed and put on ice for 10 min. To the solution was then added 15 ml of phenol saturated with TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), heated to 65°C, mixed gently and cooled on ice. After centrifugation for 30 min. at 40000 g the  
35    aqueous phase was ether extracted, ethanol precipitated and the

pellet was resuspended in TE-buffer. The DNA was further purified by banding in a CsCl density gradient (Maniatis et al., 1982).

- 5 E.coli plasmid DNA was prepared by the SDS lysis method (Maniatis et al., 1982); minipreparations of plasmid DNA for restriction enzyme analysis and transformations were prepared according to Holmes and Quigley (1981). B.subtilis plasmid DNA was prepared by the alkaline lysis method. (Maniatis et al.,  
10 1982).

d) Cloning of chromosomal DNA from Bacillus spp. PL236 into E. coli

- Restriction enzymes and T4 DNA ligase were obtained from New  
15 England Biolabs and used as described by the manufacturers. After digestion with PstI, EcoRI or HindIII restriction enzyme the DNA was heated to 65°C for 10 min. and ethanol precipitated. 10 µ of linearized pBR322, pJKK3-1 or pUN121 and 20 g of fragmented B. spp. chromosomal DNA were ligated with 5 units of  
20 DNA ligase in a final volume of 100 µl (16 h, 15°C). The ligated DNA was used to transform competent E.coli MC1000 to tetracycline resistance essentially as described by Mandel and Higa (1970). (pBR322: 20 µg/ml, pUN121: 7 µg/ml and pJKK3-1: 10 µg/ml).

25

e) Detection of cellulase-positive E.coli clones

- The detection of cellulase activity on plates was performed using a modification of the technique of Teather and Wood (1982). E.coli clones were grown overnight on solid NY medium at  
30 37°C. Cells were lysed by overlaying the colonies by topagar containing phosphate buffer (100 mM, pH 7.0), agar (0.7%) CMC (0.2%), SDS (0.25 mg/ml), and chloramphenicol (200 µ/ml) and subsequent incubation overnight at 37°C. Plates were then flooded with an aqueous solution of Congo red (1 mg/ml) for 15  
35 min. and subsequently washed with 1 M NaCl. Cellulase-positive

colonies were surrounded by a yellow halo on a red background.

f) Maxicells

Plasmid-encoded proteins were analysed using the maxicell method of Sancar et al. (1979) with the following modification. After  
5 UV irradiation, the surviving cells were killed by incubating the cells with D-cycloserine (150 µg/ml) for 48 h at 37°C.

g) Gel electrophoresis

(<sup>35</sup>S)-L-methionine labelled maxicell proteins and other proteins  
10 were analysed by electrophoresis on 15% (0.075 per cent bisacrylamide) SDS-polyacrylamide gels (Laemmli, 1970). Proteins were visualized either by staining with Coomassie Blue G 250 or by autoradiography.

15 Analysis of DNA was done by electrophoresis on agarose gels with the buffer described by Loening (1967).

h) Detection of cellulase activity in polyacrylamide gels

Detection of cellulase activity in protein bands separated by  
20 SDS-polyacrylamide gel electrophoresis was done by a modified zymogram technique described by Beguin (1983). Protein preparations were electrophoresed on a SDS-polyacrylamide gel as described above and the gel was washed 3 times 30 min. in phosphate buffer (100 mM, pH 7.0) layered on to a thin (0.8 mm)  
25 agarose gel (agarose, (1.8 per cent), CMC, (0.2 per cent), phosphate buffer, (100 mM, pH 7.0)), and incubated for 4 h at 42°C. Cellulase activity was visualized by staining the agarose gel for 30 min. in an aqueous solution of Congo red (1 mg/ml) followed by washing the gel in 1 M NaCl.

30

i) Colorimetric cellulase assay

Cellulase activity in cell extracts was analysed by measuring the increase in reducing groups released by the hydrolysis of CMC (Miller, 1959). An appropriate amount of enzyme was in-  
35 cubated with 1.5 ml of 1 per cent CMC in phosphate buffer (100

- mM, pH 7.0). After 30 min. of incubation at 55°C, 1.5 ml of dinitrosalicyclic acid reagent was added and the samples were boiled for 5 min. The absorbance was read at 550 nm against blanks containing equivalent amounts of extract from the E.coli recipient strain. One unit of cellulase released 1 nM of glucose equivalents per second by reference to a standard curve.

j) DNA-Sequencing

- Single end labelled DNA fragments were isolated and sequenced by the chemical modification method (Maxam and Gilbert, 1980). The cleavage products were separated on 8% or 20% polyacryl-amide gels and thereafter autoradiographed at -70°C using intensifying screens.
- The dideoxyribonucleotide method of Sanger et al., (1977) was used for the sequencing of Endo3A using derivatives of pUC18 (Yanisch-Perron, 1985).

k) Southern-analysis

- Chromosomal DNA from Bacillus spp. (PL236) was digested with restriction enzymes as required and fractionated on 1% (w/v) agarose gels. DNA was then blotted onto nitrocellulose filters. <sup>32</sup>P-labelled DNA probes (recombinant plasmids) were prepared by nick translation (Rigby et al., 1977) using <sup>32</sup>P- $\alpha$ -CTP (Amersham) and hybridization was carried out as described by Southern (1975). Autoradiography was performed at -70°C using intensifying screens.

l) Transformation of competent B. subtilis cells

- A modified version of Dubnau and Davidoff-Adelson's (1971) procedure for preparing competent cells of B. subtilis is used. 10 ml of LB-medium is inoculated with the strain in the morning. 7 hours later sequential dilutions in KM-1-medium are made and incubated overnight at 37°C. The following morning, the second-most diluted and growing culture is diluted ten times in KM-2-

medium. The cells are harvested after 45-60 minutes of incubation by centrifuging for 3 minutes at 7K. They are resuspended in 1/10 volume of the supernatant and 1/50 volume of 86% glycerol is added. 0.1 ml amounts are frozen on liquid nitrogen and stored at -80°C.

In order to transform these competent cells, the method of Ehrlich (1986) is used, with some modifications. BTF is prepared and preheated to 42°C. 0.01 ml of DNA is placed in a reaction vessel, and the competent cells are thawed at 42°C. BTF is added to the cells at a ratio of 1:1, and 0.1 ml of the mixture is added to the DNA. The cells and DNA incubate with shaking for 20 minutes at 37°C. A further 30 minutes of gene expression with 0.1 ml of NY-medium is needed, if kanamycin resistance is desired. The cells are finally spread on relevant plates. Recipes for stock-solutions are as follows:

Stock solutions for making *B.subtilis* competent cells

20

Salt mix: 10 mM  $\text{CaCl}_2$ , 1mM  $\text{FeCl}_3$  and 1mM  $\text{MnCl}_2$ .

10 X MM: 20 g  $(\text{NH}_4)_2\text{SO}_4$ , 60 g  $\text{KH}_2\text{PO}_4$ , 140 g  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  in 1L.

25

KM-stock: 100 ml 10 X MM, 10 ml 10% Na-citrate, 2 ml 1M  $\text{MgSO}_4$  in 1L.

KM1-stock: 960 ml KM-stock, 20 ml 20% glucose, 1 ml 20% casamino acids, 20 ml 5% yeast extract, 30 l 1mM  $\text{MnCl}_2$  in 1L.

30

KM2-stock: 960 ml KM-stock, 20 ml 20% glucose, 1 ml 20% casamino acids, 20 ml 5% yeast extract, 1 ml salt mix, 1 ml

35

25

0.5 M  $\text{CaCl}_2$ , and 0.8 ml 1M  $\text{MgCl}_2$  in 1L.

5     BCG:                   100 ml 10 x MM, 10 ml Na-citrate, 2 ml 1M  $\text{MgSO}_4$ , 1 ml saltmix, 20 ml 20% glucose in 1L.

10     BTE:                   800 ml BCG, 100 ml 10mM EGTA, 100 mM  $\text{MgCl}_2$  in 1L.  
(Prepared fresh before use).

#### ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC STRAINS

15     In order to clone genes coding for cellulose-degrading enzymes the following screening programme was set up to find suitable cellulolytic donor strains. Various compost samples were used as source of cellulolytic microorganisms.

20     Serial dilutions of compost samples were plated out on ASC agar medium and cellulolytic activity was detected through the formation of clearing zones around the colonies. Several cellulolytic bacteria were isolated. One of the most active of these isolates which was identified as Bacillus spp. PL236 (NCIMB 40250) was selected as the donor strain for the cloning  
25     experiments.

30     The strain rapidly degraded both acid swollen cellulose and microcrystalline cellulose (Avicel, Merck) on agar medium. At the optimum temperature of growth (42°C) the clearing zones appeared in 2-3 days.

35     Adding small amounts of Bacillus spp. PL236 culture to suspensions of microcrystalline cellulose makes the cellulose crystals lump together tightly and sediment.

This suggests that either the Bacillus spp. PL236 cells or the extracellular cellulase enzymes have a strong affinity for the cellulose substrate and tightly bind the cellulose crystals together.

5

#### SCREENING ASSAY FOR RECOMBINANT CLONES

The first attempts to clone cellulases from Bacillus spp. PL236 were directed towards the endocellulases genes from Bacillus spp. PL236. To facilitate the screening on plates of endocellulase positive clones, an assay using the dye Congo red (Teather and Wood, 1982) was adapted to E.coli.

In order to detect cellulase activity trapped inside the recombinant E.coli cells the cells were lysed by adding SDS to the top agar. This modification of the top agar had no measurable effect on the CMC-degrading enzymes of Bacillus spp. PL236, when the modified assay was used on this organism.

#### MOLECULAR CLONING OF ENDOCELLULASES

Several endocellulase genes were cloned from the cellulolytic Bacillus spp. strain (PL236). An endocellulase 1 clone (PL517) was made by ligating PstI partially digested PL236 chromosomal DNA with PstI cleaved pBR322 and subsequently transforming competent E.coli cells.

An endocellulase 2 clone (pPL382) was made by ligating HindIII partially digested PL236 chromosomal DNA with HindIII cleaved pJKK3-1 (an E.coli/B.subtilis shuttle-vector) and subsequently transforming competent E.coli cells.

An endocellulase 3 clone (pPL591) was made by ligating EcoRI partially digested PL236 DNA with EcoRI cleaved pUN121 and subsequently transforming competent E.coli cells.



An endocellulase 4 clone (pPL592) was made by ligating HindIII partially digested PL236 DNA with HindIII cleaved pUN121, and subsequently transforming competent E.coli cells.

5

Transformants derived from the use of both the pBR322 and the pUN121 plasmid vector were screened by their tetracycline resistance (pBR322: 20 g/ml, pUN121: 7 µg/ml and pJKK3-1: 10 µg/ml).

10

The transformants were replicated to another set of plates and overlaid by the modified CMC top agar. The plates were incubated overnight and stained with Congo red as described above.

15 Under the reisolation procedure it was observed that it was possible to detect positive clones without adding the cell lysing agent SDS to the topagar, although the diameter of the halo was considerably smaller.

20 EXPRESSION AND CHARACTERIZATION OF THE CLONED CELLULASES

Plasmids from the cellulase-positive transformants were isolated and analyzed with restriction enzymes. Restriction enzyme maps of representative plasmids from all four cloning experiments are shown in Fig. 1.

25

To determine the molecular weight of the cloned endoglucanases a zymogram technique (Beguin, 1983) was used. Total protein preparations from representative endoglucanase clones were separated on a SDS-polyacrylamide gel. The proteins in the gel were then renatured by washing out the SDS and replicated onto an agarose gel containing CMC. Renatured proteins diffuse to this activity gel and proteins representing endoglucanase activity hydrolyse the CMC in the gel. The endoglucanase bands were then visualised by staining the activity gel with Congo red

30

35

as described above.

As appears from Fig. 1, the restriction maps of the cloned DNA (as well as the molecular weight of the endoglucanases encoded by the cloned DNA) are different in the four different clones. This indicates that these four clones represent at least four different endocellulase genes from Bacillus spp. (PL236).

The detailed analysis of the different endocellulases represented by these four clones is described in the following.

#### Example 1

##### Endocellulase 1 (Endo1)

##### Physical mapping of endocellulase gene 1

In the cloning experiment described above several cellulase-positive E.coli clones were obtained, which contained different fragments of PstI partially cleaved Bacillus spp. PL236 DNA. Fig. 2 shows the restriction maps of some of these clones. The plasmids invariably contained two PstI fragments (1000 and 1350 bp.) indicating that both were necessary for synthesis of a polypeptide with cellulase activity: Nucleotide sequencing has later shown that the 500 bp. PstI fragment present on pPL217 and pPL517 (Fig. 2) contains the C-terminal part of the complete cellulase gene (data are presented in the following). The structure of this part of the B.spp. chromosome was confirmed by Southern analysis (data are presented in the following).

##### Activity measurements

Extracts from the E.coli clones containing pPL212, pPL216, pPL517 and pBR322 were prepared from overnight cultures grown in NY medium supplemented with tetracycline. Cells were concentrated 10-fold in 100 mM phosphate buffer, pH 7.0. DNase was added and the cells were ruptured by passing them twice

through a French Press (12000 lb/in). The extracts were centrifuged for 60 min. at 40000 X g and the supernatants were assayed for cellulase activity. The cell-free extract of E.coli MC1000(pPL212), E.coli MC1000(pPL517) and E.coli MC1000(pPL216) contained 8 units, 7.2 units and 0.2 units respectively of cellulase/ml of original culture volume. (Fig. 2). 1/7 of the total cellulase activity in these cultures was found in the supernatant. The E.coli MC1000(pBR322) clone showed no cellulase activity.

The high level of cellulase activity in extracts of strains carrying the plasmids pPL212 and pPL517 is most likely due to an increased transcription of the cellulase gene originating from the  $\beta$ -lactamase promoter on pBR322. It was concluded that the cellulase is expressed in the direction shown in Fig. 2.

Measurements of the viscosity of a CMC solution and of the release of reducing sugars indicated that the cloned cellulase is an endo-(1,4)- $\beta$ -glucanase.

#### Maxicell and zymogram analysis

The molecular weight of the endoglucanase protein was analysed by the maxicell technique. The plasmids pPL212, pPL216 and pBR322 were transformed into the maxicell strain CSR603 (Sancar et al., 1979) to analyse for plasmid-encoded proteins. The plasmid pPL212 gave rise to three polypeptides of 75000 D, 65000 D and 58000 D in addition to the proteins encoded by pBR322. Apart from the pBR322 proteins, no proteins encoded by the plasmid pPL216 could be detected, due to the low expression of the cellulase gene. The three polypeptides from pPL212 were tested for cellulase activity using a gel replica technique, (Beguin, 1983).

A comparison of the cellulase activity bands and the bands on

the autoradiogram from the polyacrylamide gel showed that the 75000 D and at least one of the 58000 and 65000 D protein bands had cellulase activity. It was also found the supernatant of the Bacillus spp. PL236 culture contained at least three different proteins with cellulase activity. One of these proteins comigrated with the 58000 D protein synthesized in the maxicell. Cell extracts from E.coli MC1000 (pPL212) only revealed one active band comigrating with the 58000 protein from the maxicell E.coli CSR603 (pPL212). As mentioned previously, the following sequence data showed that the plasmids pPL212 and pPL216 did not contain the entire EndoI endoglucanase gene. The 75000 D active protein seen in the maxicell E.coli CSR603 (pPL212) is thus a fusion protein, where 105 C-terminal amino acids are encoded by pBR322 sequences (Fig. 2). This fusion protein is apparently posttranslationally processed, ending up with the 58000 D mature endoglucanase. Cell extracts from E.coli MC1000 (pPL217) and E.coli MC1000 (pPL517), which contain the complete endoglucanase gene, also gave activity bands of  $M_r$  approx 58000 D and 75000 D. The 75000 D active protein from the plasmids pPL217 and pPL517, thus represents the "genuine" initial translation product from the EndoI glucanase gene, which apparently is processed more slowly than the fusion protein from pPL212. However, both the 75000 D fusion protein synthesized from pPL212 and the "genuine" 75000 D protein synthesized from pPL517 and pPL217 are processed down to a 58000 D protein with high cellulase activity.

From the sequence data it can also be predicted that the endoglucanase expressed from pPL216 is synthesized a fusion protein, where the 26 C-terminal amino acids are encoded by pBR322 sequences (Fig. 2). This protein was not detected by the maxicell technique and zymograms using extract from PL216 only revealed one active band of 58000 D, which most likely represents the processed protein. The processing in E.coli of the two fusion proteins from pPL212 and pPL216, which represent two different lengths of the C-terminal "tail" thus results, in both

case, in an active protein of approx. 58000 D. This indicates that the endocellulase is processed from the C-terminal, because N-terminal processing would result in two proteins with a difference in  $M_r$  of approx. 9000 D, which would easily have been  
5 detected on the zymograms.

It is therefore most likely that endoglucanase 1 (Endo1) is synthesized as a proenzyme which at least in E.coli (and possibly also in B.spp.) is modified by stepwise removal of  
10 approx. 150 C-terminal amino acid residues and approx. 30 N-terminal amino acid residues, corresponding to the removal of the signal peptide. The endoglucanase seems to be modified correctly as indicated by the fact that the final processing product, the 58000 D activity band present in E.coli MC1000  
15 (pPL212) extract, apparently comigrate with one of the endoglucanases present in the supernatant of cultures of Bacillus spp. PL236.

#### Temperature optimum and stability

20

The cellulase activity of the extracts was measured at different temperatures and the highest activity of Endo1 produced in E.coli was found at 60°C (Fig. 3). The heat stability of the endoglucanase was tested by incubating the extracts at 50°C,  
25 55°C and 60°C for varying periods and the residual activity was measured as outlined in Materials and Methods. Although the highest activity was observed at 60°C with a fixed incubation time of 30 min., the enzyme is inactivated at this temperature with a  $t_{1/2}$  of 1.2 h. At 50°C and 55°C no inactivation was observed  
30 after 5 h of incubation.

#### DNA-sequence

The nucleotide sequence of the endocellulase gene 1 (Endo1) was  
35 deduced from the plasmid pPL517 which contains approx. 2850 bp

of Bacillus spp. PL236 DNA.

The sequence was determined by the chemical modification method (Maxam and Gilbert, 1980) using the partial restriction map and the sequencing strategy outlined in Fig. 4.

The complete nucleotide sequence is shown in Sequence listing ID#1. A computer analysis of this sequence revealed only one open reading frame long enough to encode the approx. 75000 D protein detected by the maxicell and zymogram analysis of extracts from the cellulase-positive E.coli MC1000(pPL517). This sequence which begins at nucleotide 677 and ends at nucleotide 2776, encodes an enzyme of 700 amino acids. The  $M_r$  calculated from the DNA sequence was 77006 D.

Within the open reading frame there were three potential initiation codons (ATG at positions 677, 737 and 749), but only the ATG codon at position 677 was preceded by a ribosome binding site (AAGGAGG) (McLaughlin et al., 1981). It was therefore concluded that the ATG codon at position 677 was the correct initiation codon.

The initiation codon is followed by an amino acid sequence which resembles signal sequences found in gram-positive organisms. Such sequences consist of a relatively short hydrophilic region at the N-terminal followed by a longer sequence of hydrophobic residues.

By using the signal sequence cleavage model proposed by Heijne (1983) the cleavage site can be predicted to be between the two first alanine residues in the sequence Asn-Ala-Ala-Ala. The signal sequence is thus 31 amino acids long.

The upstream and downstream regions contained no significant homology to the consensus sequence of the sigma 43 promoter of

B. subtilis and no terminator-like sequences.

Southern analysis

5 The Bacillus spp. (PL236) chromosomal DNA was digested with  
HindIII, PstI, EcoRI and XhoI and plasmids pPL212 and pPL509  
were used as probes for the hybridization. Plasmid pPL212  
contains two PstI fragments (1350 bp. and 1000 bp.) and plasmid  
pPL509 contains only the 500 bp. PstI fragment of the entire  
10 EndoI gene, represented by the plasmid pPL517, which contains  
three PstI fragments (1350 bp., 1000 bp. and 500 bp.) of  
Bacillus spp. PL236 DNA. The pPL212 probe recognized the  
expected two PstI fragments (1350 bp., 1000 bp.) and the pPL509  
the 500 bp. PstI fragment in the Bacillus spp. PL236 PstI  
15 digest. Both the pPL212 and the pPL509 probe also recognized the  
same overlapping EcoRI fragment and the same overlapping HindIII  
fragment in Bacillus spp. PL236, EcoRI and HindIII digest. These  
results indicate that the Bacillus spp. PL236 DNA insert in  
pPL517 was cloned in a non-deleted form and that the three PstI  
20 fragments in pPL517 are continuous on the Bacillus spp. PL236  
chromosome.

Expression of the EndoI gene in B.subtilis

25 For the cloning experiments in B.subtilis, pPL517 was used as  
the donor of the EndoI gene and pDN2801, carrying a strong  
Bacillus promoter P<sub>m</sub>, was used as the Bacillus vector (Fig. 5).

The EndoI gene-containing EagI fragment was ligated to EagI  
30 cleaved pDN2801 and by subsequent transformation to competent  
B.subtilis cells (DN1885), strain CH7 was obtained. To test  
whether the processed C-terminal part was necessary for the  
expression of the EndoI gene in B.subtilis cells, a construc-  
tion was made where the EndoI gene was fused to vector sequen-  
35 ces in the internal BglII site. This fusion replaces the coding

region for the C-terminal 94 amino acids with 55 "random" amino acids encoded by vector sequences.

5 Similar constructions made in E.coli vectors, though fused to different vector sequences, resulted in an active periplasmic endoglucanase in E.coli, which was processed in the "correct" manner. Part of the Endo1 gene contained in the BglII fragment from pPL517 was ligated with BamHI cleaved pDN2801 and subsequent transformation to competent B. subtilis cells (DN1885) 10 resulted in strain CH14.

Transformants were in both cases screened for their chloramphenicol resistance, and the desired plasmid constructions in the strains CH7 and CH14 by restriction analysis of their plasmids. 15 The two versions of the Endo1 gene on the plasmids pCH7 and pCH14 are thus transcribed from the same promoter  $P_m$ . A restriction map of the plasmids is shown in Fig. 6.

The B.subtilis DN1885 used for these experiments produces an 20 endoglucanase of its own, which of course gave some background activity. The Endo1 gene product was exported to the culture medium from the recombinant strain CH7, and the activity measured in the culture supernatant was approximately 20 times higher than the background activity (Fig. 6). No extracellular 25 activity above the background level was detected from the recombinant strain CH14 which contains the Endo1 gene with the substituted C-terminal.

The culture supernatants from the strains CH7 and CH14 and cell 30 extract from CH7 cells were analysed by the zymogram technique. The zymogram revealed active protein bands of approx. 75000 D and 58000 D from the CH7 cell extract and only one active protein band of approx. 58000 D from the CH7 culture supernatant. These bands correspond to those observed in E.coli and the 35 processing of approx. 90 amino acids from the C-terminal appears



to take place in B.subtilis too.

The plasmid pCH7 was transformed to PL1801, which is a derivative of DN1885 lacking the two main exoproteases (apr<sup>-</sup>, npr<sup>-</sup>), resulting in the strain CH14. The Endol cellulase as produced from CH14 was processed "normally" indicating that the two main exoproteases from B.subtilis are not responsible for the C-terminal processing of the Endol cellulase.

A very weak active band of approx. 58000 D was detected from the CH14 culture supernatant, indicating that the manipulated gene is expressed and processed in at least almost the same way as the native gene product. Among other things, the very low expression from pCH14 and the fact that the two genes are expressed from the same expression signals may indicate that the approx. 90 C-terminal amino acids are necessary for the export of the Endol gene product from B.subtilis.

#### Optimization of expression of the Endol gene in E. coli

In order to optimize the expression of Endol, the Endol gene was combined with the strong E.coli promoters P<sub>R</sub> and P<sub>L</sub> originating from phage lambda (Remaut et al., 1981). Both promoters are repressible by the lambda cI857 repressor, which is heat labile, thus rendering the P<sub>R</sub> and P<sub>L</sub> promoters heat inducible, in cells producing the lambda cI857 gene product. (Ptashne et al., 1982).

The P<sub>R</sub> promoter is contained on the expression plasmid pPL170 together with the lambda cI857 gene. (Fig. 7; Jørgensen, 1983).

The P<sub>R</sub> promoter was placed upstream of the Endol gene by ligating the P<sub>R</sub> containing PvuI - SalI fragment from pPL170, to the Endol gene containing PvuI - SalI fragment. Transformation to competent MC1000 cells resulted in the strain TL05 containing the plasmid pTL05. In the plasmid pTL05 the  $\beta$ -lactamase promoter

is deleted, thus bringing the Endo1 gene under transcriptional control of the  $P_R$  promoter. At this point, the Endo1 gene was believed to be contained within the BglII fragment from pPL2129. The Endo1 gene fusion to vector sequences on pPL212 was therefore transferred to pTL05, resulting in a fusion protein where 105 C-terminal amino acids are encoded by vector sequences. This fusion protein is however processed correctly as shown earlier with the strain PL212. The cellulase production from TL05 is completely repressed at 28°C and induced at 42°C.

10

The  $P_i$  promoter provided on the plasmid pPLc28 (Remaut et al., 1981) was combined with the Endo1 gene by ligating the BglII fragment from pPL212 to BamHI cleaved pPLc28. Transforming to competent PL248 cells, which are harbouring the lambda cI857 gene on a compatible pACYC177 based plasmid (pNF2690), resulted in the strain LA03, containing the plasmid pLA03 (Fig. 7). The Endo1 gene is thus fused to vector sequences, but due to unspecified DNA sequences in pPLc28 the length and nature of the resulting fusion protein is unknown. The cellulase production from LA03 was completely repressed at 28°C and induced at 40.5°C.

20

The cellulase production from LA03 and TL05 was evaluated at different temperatures.

25

LA03, TL05 and PL212 were grown overnight at 28°C in NY medium supplemented with the appropriate antibiotics (AMP + KAN, KAN and TET, respectively). For each strain the overnight cultures were diluted 100 fold in NY medium (AMP + KAN, KAN and TET, respectively), and the diluted cultures were grown at different temperatures between 28°C and 42°C. Cells from each culture were harvested at  $OD_{450} = 1$  and lysed on a French Press, and the activity in the extracts was determined as described earlier.

30

LA03 which exhibited the highest cellulase production was unable

35

to grow at temperatures above 40.5°C. The experiment was repeated without antibiotic selection pressure in the diluted cultures. Similar results were obtained, but LA03 grew very slowly at temperatures above 40.5°C. However, this growth was  
5 followed by a significant loss of the plasmid pLA03. No significant loss of pLA03 at temperatures up to 40.5°C or pPL212 and pTL05 at any temperature, was observed.

#### Optimization of expression of the Endol gene in B.subtilis

10

In order to optimize the expression of the Endol gene in B.subtilis, the Endol gene was fused to the expression-signals (promoter, ribosome binding site and signal sequence) from the alpha-amylase gene from B.licheniformis, which is expressed in  
15 high amounts in B.subtilis.

pPL1759 contains the promoter, ribosome binding site and most of the signal peptide of the B.licheniformis alpha-amylase (Stephens et al., 1984). The downstream side of this region ends  
20 with a PstI site, which again is followed by a polylinker (Fig. 8). Between the PstI and the SalI site in the polylinker of pPL1759, a synthetic DNA fragment consisting of two complementing oligonucleotides creating PstI and SalI "sticky" ends was inserted. In the resulting plasmid pCH52 the synthetic  
25 linker reconstitutes the missing part of the signal peptide of the alpha-amylase and further encodes the first 14 N-terminal amino acids of the mature Endol cellulase (Fig. 8). The linker thus creates a hybrid signal peptide cleavage site between the alpha-amylase and the cellulase. The expected cleavage site is  
30 shown in Fig. 8. From pPL517 the Endol gene was excised without promoter on an EagI fragment and inserted into the unique EagI site in pCH52. The plasmid in which the Endol gene was inserted in the correct orientation was named pCH54.

35 pCH54 contains two direct repeated sequences of 45 bp (e.g. the

45 N-terminal base pairs of the mature Endo1 gene) which may recombine, deleting the region between them (Ehrlich et al., 1986). This recombination event, however, occurs with a very low frequency when the repeat is as small as 45 bp. In order to  
5 enrich the amount of plasmid that has recombined, a plasmid preparation of pCH54 was cut with the enrichment restriction enzyme KpnI. pCH54 contains a unique KpnI site between the two direct repeats and only non-recombinant plasmids are cut with KpnI, while recombinant plasmids stay circular. When B.subtilis  
10 (DN 1885) was retransformed with this mixture, transformants were mostly (90%) containing recombinant plasmids, since B.subtilis competent cells are not transformed with linerized plasmid DNA. The recombinant plasmid was called pCH57 and is contained in the strain CH57. The structure was confirmed by  
15 restriction analysis, but the gene fusion was not confirmed by DNA sequencing. In this construction pCH57 the Endo1 gene is thus perfectly fused to the alpha-amylase expression signals.

The endoglucanase is produced extracellularly from the B.  
20 subtilis strain CH57, indicating that the hybrid signal cleavage site is functioning. The secreted Endo1 endoglucanase is processed to the expected  $M_r$ , namely 58000 D.

The production of the Endo1 cellulase from CH57 was evaluated in  
25 two different media, NY (overnight at 37°C) and BPX (7 days at 37°C). The BPX medium is a very rich medium in which the nutrients are slowly released, thus keeping the cells in an early stationary fase for several days during fermentation. The alpha-amylase expression signals function particularly well in  
30 this medium. The results appear from Table 1 below.

Table 1

5	STRAIN	PLASMID	U/ml	U/ml
			NY	BPX
10	CH7	pCH7	6.5	55.0
	CH57	pCH57	13.0	325.0
	DN1885	---	0.3	40.0

15 Analysis of the culture supernatant (BPX-medium) on PAGE revealed a dominant (90%) endoglucanase band corresponding to a concentration of endoglucanase in the supernatant of approx. 0.5 g/L.

20 Example 2

Endocellulase 2 (Endo 2)

DNA-sequence

25

The nucleotide sequence of endocellulase 2 (Endo2) was deduced from the plasmid pPL382 which is described above. The plasmid contains approx. 2500 bp. of Bacillus spp. PL236 DNA. The sequence was determined by the chemical modification method (Maxam and Gilbert, 1980) using the partial restriction map and the sequencing strategy outlined in Fig. 9.

30

The complete nucleotide sequence is shown in Sequence Listing ID#3. A computer analysis of this sequence revealed only one open reading frame long enough to encode for the approx. 56000 D protein detected in the zymogram analysis of the extract from

35

the cellulase positive clone E.coli MC1000(pPL382). This sequence begins at position 172 and ends at 1869 and encodes an enzyme consisting of 566 amino acids. The calculated  $M_r$  is 62551 D which is slightly higher than the  $M_r$  of 56000 D determined by  
5 zymogram analysis. This difference could be due to inaccuracy in the zymogram analysis or to post-translational processing beyond the expected processing of the signal peptide. The ATG initiation codon in position 172 was selected because it was the only initiation codon within the open reading frame, which was  
10 proceeded by a ribosome binding site AAGGAGG (McLaughlin et al., 1981).

This initiation codon was followed by a signal sequence-like sequence, and by use of the signal sequence cleavage model  
15 proposed by Heijne (1983), the cleavage site could be predicted to be between the two alanine residues in the middle of the sequence Leu-Ala-Ala-Ala. The signal sequence of the Endo12 is thus 30 amino acids long.

20 The region upstream of the open reading frame contained a sequence homologous with the sigma 43 type promoters of B.subtilis (Johnson et al., 1983) at position 46-75.

This sequence consists of TTTACA as the -35 region and TATTAT as  
25 the -10 region; the two are separated by 18 nucleotides.

A palindromic repeat sequence of 13 bp. was found downstream of the termination codon at position 1956-1981, which seems to resemble a rho-independent terminator (Rosenberg and Court,  
30 1979).

#### Southern analysis

The Bacillus spp. (PL236) chromosomal DNA was digested with  
35 HindIII, PstI, EcoRI and XhoI and the plasmid pPL382 was used as

a probe for the hybridization. The hybridization pattern obtained confirmed that the Bacillus spp. PL236 DNA was cloned in non-deleted form, that the two HindIII fragments from pPL382 was continuous on the Bacillus spp. PL236 chromosome and that the Endo2 gene was different from the other cloned endoglucanases.

#### Expression of Endo2 in B.subtilis

10 Plasmid pPL382 was transformed to B.subtilis DN1885 to achieve secretion of the mature Endo2 product. B. subtilis DN1885 (pPL382) was grown aerobically in 640 ml LB-medium containing 10  $\mu$ g/ml tetracycline for 30 hours. The supernatant was concentrated by precipitation for 24 hours with  $(\text{NH}_4)_2\text{SO}_4$  at 70% saturation. After 5 hours of dialysis against 100 mM Tris-HCl pH 7, the concentrated supernatant was heated to 55°C for 15 minutes. Denatured protein was removed by centrifugation and the soluble proteins were subsequently precipitated with  $(\text{NH}_4)_2\text{SO}_4$  at 70% saturation. The resolubilized proteins were dialyzed against 20 100 mM Tris-HCl pH 7 with a final volume of 2 ml and were applied to a 80 cm x 1 cm gel filtration column containing Ultrogel AcA 44 (LKB). Active fractions were pooled, concentrated and applied to a SDS containing polyacrylamide gel, where the endoglucanase appeared as a single band at 56 kDa. The 25 activity yield of the method was approximately 10%.

The endoglucanase comigrates with the endoglucanase obtained from extracts of MC1000 (pPL382) as detected by Zymogram analysis in the supernatant of DN1885 (pPL382). The endoglucanase activity of DN1885 (pPL382) is about 25 times that of 30 strain DN1885 when grown in NY medium.

EXAMPLE 3Endocellulase 35 Physical mapping of the endocellulase clone 3

- The endocellulase clone 3 is represented by the plasmid pPL591 which contains a 11000 bp. EcoRI fragment of Bacillus spp. PL236 DNA. A partial restriction map of this plasmid is shown in Fig.
- 10 10. Zymogram analysis of extracts from E.coli MC1000(pPL591) shows that the insert gives rise to four proteins with cellulase activity. The approx.  $M_r$  of these proteins were 60000 D, 56000 D, 45000 D and 30000 D (Fig. 10).
- 15 Southern analysis showed that the EcoRI fragment from pPL591 was cloned in a non-deleted form from the Bacillus spp. PL236 chromosome, and indicates that the DNA did not contain the DNA-sequences encoding the Endo1 and Endo2 genes.
- 20 To analyse whether these proteins represented post-translational processing products from one or several cellulase genes, deletion plasmids were made using the restriction enzymes HindIII and SmaI. Deletion of the 4800 bp. SmaI fragment, resulting in the plasmid pPL538, did not eliminate any of the
- 25 four cellulase bands on the zymogram. The B.spp. DNA insert on pPL591 contained 5 HindIII sites. Digestion of pPL591 with HindIII thus gave 6 fragments of 6600 bp. (vector fragment), 3200 bp., 1700 bp., 1550 bp., 1350 bp. and 900 bp., where the 1700 bp. fragment had originated from the HindIII site within
- 30 the pUN121 vector plasmid (Fig. 11).

- Elimination of all of the HindIII fragments except the 1700 bp. fragment (reinserted in the opposite direction) resulted in the plasmid pPL540. Removal of the rest of the Bacillus spp. PL236
- 35 DNA by eliminating the EcoRI fragment from pPL540 resulted in



the plasmid pPL587. Both the E.coli MC1000(pPL540) and the E.coli MC1000(pPL587) were cellulase-positive and zymogram analysis of extracts from these clones revealed only the 45000 D and 30000 D proteins. The protein-coding capacity of the Bacillus spp. PL236 DNA (approx. 1500 bp.) is approx. 55000 D, which is too small to contain two endoglucanase genes of 45000 D and 30000 D. The 30000 D protein on the zymogram is thus most likely a result of immature post-translational processing of the 45000 D protein. The cellulase gene encoding the 45000 D protein was designated Endo3A. The Endo3A gene was cloned in both directions on the plasmids pPL587 and pPL538 giving rise to the same two proteins, thus eliminating the chance of the protein being a fusion protein.

Elimination of the 1550 bp., the 1350 bp. and the 900 bp. fragment resulted in the plasmid pPL542. Extracts from E.coli MC1000(pPL542) revealed cellulase positive proteins of approx. 30000 D, 45000 D, 49000 D and 56000 D (Fig. 10). From these preliminary results, the existence of two additional endoglucanase genes within the original insert on pPL591 and pPL538 are postulated. The additional endoglucanase genes are designated Endo3B and Endo3C. Their postulated position on the Bacillus spp. PL236 DNA is shown in Fig. 11. The postulated model is based on the assumption that the 60000 D protein made from pPL538 and pPL591 is converted to a truncated fusion protein of 49000 D made from pPL542 where the HindIII fragment of 1350 bp. is deleted.

#### DNA-sequence of Endo3A

The DNA sequence of the Endo3A gene was deduced from the plasmid pPL540 containing approx. 1500 bp. of Bacillus spp. PL236 DNA using the dideoxy chain termination method. The gene was placed in pUC18 in both orientations, and a number of deletions were constructed. Standard primers were used except for one synthe-

tic oligonucleotide that was used for sequencing a region with no practical restriction sites.

The C-terminal part of the gene was deduced from the plasmid pPL538. The partial DNA-sequence is shown in Sequence Listing ID#6. The sequence revealed an open reading frame coding for a protein with a  $M_r$  of about 62000 D which is in agreement with the observed protein of 60kD in the zymograms. The ATG start codon (position 30) is preceded by a typical ribosome binding site (McLaughlin et al., 1981). The initiation codon is followed by a typical gram-positive signal sequence and by using the signal sequence cleavage model (Heijne, 1983) a signal sequence of 36 amino acids is revealed.

#### 15 EXAMPLE 4

##### Endocellulase 4

##### Zymogram analysis

20

The endocellulase clone No. 4 is represented by the plasmid pPL592, which contains approx. 14000 bp. of Bacillus spp. PL236 DNA. A partial restriction map is shown in Fig. 1.

25 A zymogram analysis of extracts from E.coli MC1000(pPL592= revealed three cellulase active proteins with  $M_r$  values of approx. 92000 D, 74000 D and 71000 D. Further analysis is necessary to determine whether these proteins are encoded by one or several cellulase genes.

30

Southern analysis confirmed the origin of the cloned DNA on the Bacillus spp. PL236 chromosome, and indicates that the cloned DNA is not represented on the other endoglucanase clones.

35

EXAMPLE 5A. Endol cloned and expressed in Bacillus subtilis

5 An agar slant was inoculated with B. subtilis strain CH 57 and  
incubated for 20 hours at 37°C. 10 ml of a 0.9 % NaCl-solution  
was added to the test tube which was shaken to suspend the  
cells. The cell suspension was used to inoculate a 2 l  
fermentor.

10

The following parameters were used to run the fermentation:

Temperature: 37°C.

15 Aeration: 1,1 l/minute.

Stirring: 1100 rpm.

Fermentor: A 2 l model with a working volume of 1,5 l.

20

The pH was maintained between 6.2 and 7.2 for the first 40 hours  
of fermentation. After that the pH was maintained between 6.7  
and 7.2. The pH was maintained within this range by dosing with  
NH<sub>3</sub> and H<sub>3</sub>PO<sub>4</sub>.

25

Dosing of a glucose solution was initiated after 40 hours at a  
flow rate of 3.7 ml/hour.

Substrate

	Potato starch degraded with Termamyl*	50 g
	Soybean meal	110 g
5	Corn steep Liquor	16.5 g
	Alburex (potato protein)	27.5 g
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.2 g
	KH <sub>2</sub> PO <sub>4</sub>	1,2 g
	Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	5,9 g
10	Water added up to 1100 ml.	

\*Termamyl is a commercial B. licheniformis  $\alpha$ -amylase available from Novo Nordisk A/S.

15 Glucose solution

	Glucose.H <sub>2</sub> O	600 g
	Citric acid	0,6 g
	Water added up to 1000 ml	

20

The fermentation was stopped after 166 hours of fermentation at an OD<sub>650</sub> value of 122. At that time there were 1200 ml of fermentation broth in the fermentor.

- 25 The fermentation broth was centrifuged, and the extracellular volume was 400 ml containing 40 CMC-endoase units per ml (16.000 CMC-endoase units in all). The culture medium was further processed by filtration and dilution followed by concentration on an Amicon ultrafiltration module with a cut-off at 10.000 MW.
- 30 The concentrated enzyme solution was frozen.

- Half of the frozen liquid was thawed and diluted with deionized water and then concentrated once more on an Amicon ultrafiltration module. The total yield was 4471 CMC-endoase
- 35 units (from 8000 CMC-endoase units).

The total volume of 1050 ml was subjected to ion exchange chromatography at pH 7. The enzyme was bound to a DEAE-Sephacryl anion exchange column (300 ml volume) at pH 7 (50 mM tris-HCl). The Endol enzyme was eluted at pH 7 with 0.3 M NaCl.

The purified enzyme has a molecular weight of 58,000 D on SDS-PAGE. The pI is 4.0. Its activity is 30 CMC-endoase units per mg protein.

The protein determination is based on the amino acid composition of the enzyme deduced from the DNA sequence: 13 tryptophan, 30 tyrosine and a molecular weight of 57,566 D. The extinction coefficient is calculated by means of the following formula:

$$(13 \times 5559 + 30 \times 1197) / 57566 = 1.88$$

The purified enzyme has an endoglucanase activity of 57 CMC-endoase units-per ml and an absorbance at 280nm of 3.6.

Thus,  $(57 \times 1.88) / 3.6 = 30$  CMC-endoase units per mg protein.

#### B. Stability of Endol in detergents

The following 4 detergent compositions were used:

1. USA liquid detergent: 2 gram per liter of 6° hardness water (1 part tap water to 2 parts deionized water). The pH was measured to 7,29.
2. USA Heavy Duty Powder detergent: 0,9 gram per liter of 6° hardness water. The pH was measured to 9,2.
3. Heavy Duty Powder detergent (2) with bleach and activator: 0.12 gram/l Na-perborate tetrahydrate and 0,088 gram/l NOBS. The pH was measured to 9,2.

4. European Heavy Duty Powder detergent with bleach and activator (Batch DR 8806 Europe). 5 gram per liter in 9° hardness water.

5

Celluzyme™ (batch CAX 007 crude enzyme with cellulase and other enzymes) 2353 CMC-endoase units per gram was compared with Endol 30000 CMC-endoase units per gram.

10 The enzymes were diluted to 3 CMC-endoase units per ml in all 4 detergent solutions: The endoglucanase activity after dilution was measured as described above (by determining the decrease in the viscosity of CMC). The endoglucanase activity after 60 min. incubation at 40 °C was measured and compared with the initial  
15 activity. The following results were obtained:

Detergent solution	1	2	3	4
Celluzyme™	89%	75%	66%	75%
<u>Endol</u>	106%	90%	97%	98%

20

The standard deviation is 10%.

25

It appears from the table that Endol is more stable at a pH of 9-10 compared with Celluzyme™ in these detergents.

#### EXAMPLE 6

30

#### A. Preparation of full-length (~75 kD) Endol in B. subtilis

10 l of LB medium containing 1 mM CuCl<sub>2</sub> and 10 µg/ml chloramphenicol was inoculated with 10 ml of an overnight  
35 culture of B. subtilis DN969 (B. Diderichsen et al.,

J. Bacteriol. 172(8), 1990, pp. 4315-4321) containing the plasmid pCH7 (described above in example 1), divided among 10 sterile 2 l flasks and incubated with vigorous shaking for 36 hours at 37°C. The culture was centrifuged for 10 minutes at 10000xg and 4°C after which EDTA, pH 8, was added to the supernatant to a final concentration of 5 mM.

25 g of Avicel PH-105 which had been hydrated in ethanol and washed with distilled water was added to the supernatant which was left standing with gentle stirring for 2 hours at 4°C. The supernatant/Avicel mixture was centrifuged for 10 minutes at 10000xg and 4°C. The cleared supernatant was decanted off immediately after the rotor had stopped.

The Avicel/enzyme cake was resuspended and washed in 200 ml of 1mM EDTA, and the mixture was centrifuged for 1 minute at 10000xg. This procedure was repeated twice. The Avicel/enzyme cake was then resuspended in 150 ml (1% triethylamine and 1 mM EDTA) and was left standing with vigorous stirring for 1 hour at 4°C. The mixture was centrifuged for 1 minute at 10000xg and 4°C. The supernatant was retained. This procedure was repeated twice.

The solution of enzyme and triethylamine (about 300 ml) was evaporated in vacuo to 100 ml. The temperature of the solution was not allowed to exceed 10°C. The pH was adjusted to 7 by adding 1M HCl, and the solution was frozen at -70°C.

The amount of protein in the 100 ml enzyme solution was determined to be 40 mg by means of a Bradford reagent (available from BioRad) using bovine serum albumin as the standard.

#### B. Characterisation of the ~75 kD EndoI

The enzyme obtained above had a purity of about 90%. The enzyme

was found to have a molecular weight of 75 kD on SDS-PAGE.

In immunoprecipitation experiments (carried out by rocket immunoelectrophoresis in agarose gel as described by N. Axelsen et al., Chapter 2 in A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publ. 1973), ~75 kD Endo1 was immunoreactive with a monospecific rabbit antibody raised against the core region (~58 kD form) of Endo1.

The ~75 kD and ~58 kD (processed) forms of Endo1 were tested for their ability to bind to cellulose (Avicel). 10 µg of each of ~75 kD Endo1 and ~58 kD Endo1 were added to 50 µl of a cellulose suspension (0.1% w/w Avicel, 5 mM EDTA, pH 8.0). The suspensions were shaken for 30 minutes, and the Avicel was harvested at 10000 rpm for 2 minutes. The amount of Endo1 cellulase remaining in the supernatant was analysed by SDS-PAGE. More than 95% of the ~75 kD Endo1 was bound to the cellulose, while less than 5% of the ~58 kD Endo1 was similarly bound. This shows that the C-terminal part of the ~75 kD Endo1 cellulase comprises a cellulose-binding domain.

Extensive amino acid sequence homology was found between this region (from amino acid 554 to 700) of the Endo1 cellulase and other cellulases, e.g. an endocellulase from Bacillus subtilis (Nakamura et al., 1987), the middle part of the bifunctional cellulase from Caldocellum saccharolyticum (D.J. Saul et al., Nucl.Acids Res. 17, 1988, p. 439), two endocellulases from Clostridium stercoarium (W. Schwarz et al., Biotech.Lett. 11, 1989, pp. 461-466).

#### C. Colour clarification effect of ~75 kD Endo1

The colour clarification effect of ~75 kD Endo1 was determined by exposing a prewashed worn textile surface to the enzyme and then measuring the clarity of the surface colour compared to the



clarity of the surface colour of textiles which had not been treated with the enzyme.

Black 100% cotton swatches (15 x 10 cm) were prewashed and  
5 tumble-dried under the following conditions

Detergent: Keminus (available from Irma A/S),  
1.5g/l  
Temperature: 70°C  
10 Washing time: 60 minutes  
Drying time: 30 minutes  
No. prewashing/  
drying treatments: 15

15 The swatches were prewashed in a conventional washing machine  
(Miele Deluxe Electronic W761). After each wash, the swatches  
were dried in a tumble-drier. The visual effect of the  
prewashing/drying was that the surface colour turned greyish due  
to the presence of damaged cellulose fibres causing the worn  
20 look.

After prewashing, the swatches were washed in a Terg-O-Tometer  
(toploaded mini washing machine) under the following conditions

25 Liquid volume: 800 ml  
Agitation: 100 movements/minute  
Detergent: Standard detergent, 5g/l  
Washing time: 30 minutes  
Washing tempera-  
30 ture: 40°C  
No. of swatches: 2  
~75 kD Endo1  
dosage: 0 and 60 CMC endoase units/l  
pH: 7.0  
35 No. of treatments: 3

## Standard detergent:

	LAS NANSÁ 1169/P:	10%
5	AE Berol 160:	15%
	Ethanol, 96%:	10%
	TEA:	5%
	Water:	60%

- 10 After each wash, the swatches were rinsed in tap water and dried at room temperature.

The surface colour of the swatches was analysed by measuring reflected light. White light was projected onto the surface, and  
 15 the reflection/remission was measured at 16 wavelengths (400 nm - 700 nm). The results from the measurements were processed (by means of an "Elrepho 2000" apparatus available from Datacolor, Switzerland) into Hunter coordinates of which the L-coordinate represents the grey scale values. Each swatch was analysed twice  
 20 on each side, and the results shown below are a total average from the measurements of the two swatches from the same treatment. In the table, white is L = 100, and black is L = 0.

	Dosage	0 CMC endoase/160 CMC endoase/1	
25	L	16.78	15.50
	S.D.	0.08	0.03
	Delta L	-	1.28

Comparable results were obtained with Celluzyne™ (batch PPC 2174  
 30 containing a mixture of enzymes from Humicola insolens, DSM 1800)

	Dosage				
	(CMC endoase/1)	0	15	30	60
35	Delta L	-	1.02	1.48	1.90

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Novo Nordisk A/S
- (ii) TITLE OF INVENTION: An Enzyme Exhibiting Cellulase Activity
- 10 (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: Novo Nordisk A/S, Patent Department
- (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: DENMARK
- (F) ZIP: DK-2880
- (v) COMPUTER READABLE FORM:
- 20 (A) MEDIUM TYPE:
- (B) COMPUTER:
- (C) OPERATING SYSTEM:
- (D) SOFTWARE:
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- 30 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: DK 164/90
- (B) FILING DATE: 19-JAN-1990
- (viii) ATTORNEY/AGENT INFORMATION:
- 35 (A) NAME: Thalsø-Madsen, Kine Birgit
- (B) REGISTRATION NUMBER:
- (C) REFERENCE/DOCKET NUMBER: 3425.204-WO
- (ix) TELECOMMUNICATION INFORMATION:
- 40 (A) TELEPHONE: +45 4444 8888
- (B) TELEFAX: +45 4449 3256
- (C) TELEX: 37304
- 45 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2977 base pairs
- 50 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear



59

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Bacillus lautus*

(B) STRAIN: NCIMB 40250

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 677..2776

(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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	TCCATGGCGG TAATCAGCGC TTCCGACTGC AGTACAGAGC GGCCGATACA AATGCAGCCG	180
20	ACAACCAGAT CAAGCCGTCC TTCAACATCA AAAACAACGG TACTTCGGCT GTTGATTAA	240
	GCACGCTCAA AATCCGCTAC TACTTCACCA AGGATGGTTC TGCGGCGGTG AACGGCTGGA	300
25	TCGACTGGGC GCAGCTCGGC GGCAGCAACA TTCAGATCTC GTTTGGCAAC CATACTGGCA	360
	CGAATTCCGA TACGTACGTG GAGCTGAGCT TCTCGTCCGA GGCAGGCTCG ATTGCGGCGG	420
	GCGGCCAATC CGGTGAAATC CAGCTGCGCA TGTCCAAGAC GGA CTGGTCG AACTTTAACG	480
30	AGGCGAACGA CTA CTGTTT GATGGGACGA AGACGGCCTT TGCTGACTGG GATCGGGTCG	540
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	CTGGGAGGGA TCAAAA ATG AAG ACA AGA CAA AGA AAG CGG CTG TTC GTC	709
	Met Lys Thr Arg Gln Arg Lys Arg Leu Phe Val	
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40	AGT GCG GCG CTG GCA GTA TCC TTG ACA ATG ACC GTA CCG ATG CCC GCT	757
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45	TCT GTA AAT GCA GCT GCG AGT GAT GTC ACT TTC ACG ATT AAT ACG CAG	805
	Ser Val Asn Ala Ala Ala Ser Asp Val Thr Phe Thr Ile Asn Thr Gln	
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50	TCG GAA CGT GCA GCG ATC AGC CCC AAT ATT TAC GGA ACC AAT CAG GAT	853
	Ser Glu Arg Ala Ala Ile Ser Pro Asn Ile Tyr Gly Thr Asn Gln Asp	
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	CTG	AGC	GGG	ACG	GAG	AAC	TGG	TCA	TCC	CGC	AGG	CTC	GGA	GGC	AAC	CGG	901
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5	CTG	ACG	GGT	TAC	AAC	TGG	GAG	AAC	AAC	GCA	TCC	AGC	GCC	GGA	AGG	GAC	949
	Leu	Thr	Gly	Tyr	Asn	Trp	Glu	Asn	Asn	Ala	Ser	Ser	Ala	Gly	Arg	Asp	
					80					85					90		
10	TGG	CTT	CAT	TAC	AGC	GAT	GAT	TTT	CTC	TGC	GGC	AAC	GGT	GGT	GTT	CCA	997
	Trp	Leu	His	Tyr	Ser	Asp	Asp	Phe	Leu	Cys	Gly	Asn	Gly	Gly	Val	Pro	
				95					100					105			
15	GAC	ACC	GAC	TGC	GAC	AAG	CCG	GGG	GCG	GTT	GTT	ACC	GCT	TTT	CAC	GAT	1045
	Asp	Thr	Asp	Cys	Asp	Lys	Pro	Gly	Ala	Val	Val	Thr	Ala	Phe	His	Asp	
			110					115					120				
20	AAA	TCT	TTG	GAG	AAT	GGA	GCT	TAC	TCC	ATT	GTA	ACG	CTG	CAA	ATG	GCG	1093
	Lys	Ser	Leu	Glu	Asn	Gly	Ala	Tyr	Ser	Ile	Val	Thr	Leu	Gln	Met	Ala	
	125					130						135					
25	GGT	TAT	GTG	TCC	CGG	GAT	AAG	AAC	GGT	CCA	GTT	GAC	GAG	AGT	GAG	ACG	1141
	Gly	Tyr	Val	Ser	Arg	Asp	Lys	Asn	Gly	Pro	Val	Asp	Glu	Ser	Glu	Thr	
	140				145					150						155	
30	GCT	CCG	TCA	CCG	CGT	TGG	GAT	AAG	GTC	GAG	TTT	GCC	AAA	AAT	GCG	CCG	1189
	Ala	Pro	Ser	Pro	Arg	Trp	Asp	Lys	Val	Glu	Phe	Ala	Lys	Asn	Ala	Pro	
					160					165					170		
35	TTC	TCC	CTT	CAG	CCT	GAT	CTG	AAC	GAC	GGA	CAA	GTG	TAT	ATG	GAT	GAA	1237
	Phe	Ser	Leu	Gln	Pro	Asp	Leu	Asn	Asp	Gly	Gln	Val	Tyr	Met	Asp	Glu	
				175					180					185			
40	GAA	GTT	AAC	TTC	CTG	GTC	AAC	CGG	TAT	GGA	AAC	GCT	TCA	ACG	TCA	ACG	1285
	Glu	Val	Asn	Phe	Leu	Val	Asn	Arg	Tyr	Gly	Asn	Ala	Ser	Thr	Ser	Thr	
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	205					210					215						
50	ACG	CAT	CCA	AGG	ATT	CAT	CCG	GAG	CAG	TTA	CAA	GCG	GCA	GAA	CTC	GTC	1381
	Thr	His	Pro	Arg	Ile	His	Pro	Glu	Gln	Leu	Gln	Ala	Ala	Glu	Leu	Val	
	220				225					230						235	
55	GCT	AAG	AGC	ATC	GAC	TTG	TCA	AAG	GCG	GTG	AAG	AAC	GTC	GAT	CCG	CAT	1429
	Ala	Lys	Ser	Ile	Asp	Leu	Ser	Lys	Ala	Val	Lys	Asn	Val	Asp	Pro	His	
					240					245					250		
60	GCC	GAA	ATA	TTC	GGT	CCT	GCC	CTT	TAC	GGT	TTC	GGC	GCA	TAT	TTG	TCT	1477
	Ala	Glu	Ile	Phe	Gly	Pro	Ala	Leu	Tyr	Gly	Phe	Gly	Ala	Tyr	Leu	Ser	
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61

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5	TTT ATC GAT TAC TAT CTG GAT CAG ATG AAG AAT GCT CAT ACG CAG AAC	1573
	Phe Ile Asp Tyr Tyr Leu Asp Gln Met Lys Asn Ala His Thr Gln Asn	
	285 290 295	
10	GGC AAA AGA TTG CTC GAT GTG CTG GAC GTC CAC TGG TAT CCG GAA GCA	1621
	Gly Lys Arg Leu Leu Asp Val Leu Asp Val His Trp Tyr Pro Glu Ala	
	300 305 310 315	
15	CAG GGC GGA GGC CAG CGA ATC GTC TTT GGC GGG GCG GGC AAT ATC GAT	1669
	Gln Gly Gly Gly Gln Arg Ile Val Phe Gly Gly Ala Gly Asn Ile Asp	
	320 325 330	
20	ACG CAG AAG GCT CGC GTA CAA GCG CCA AGA TCG CTA TGG GAT CCG GCT	1717
	Thr Gln Lys Ala Arg Val Gln Ala Pro Arg Ser Leu Trp Asp Pro Ala	
	335 340 345	
25	TAC CAG GAA GAC AGC TGG ATC GGC ACA TGG TTT TCA AGC TAC TTG CCC	1765
	Tyr Gln Glu Asp Ser Trp Ile Gly Thr Trp Phe Ser Ser Tyr Leu Pro	
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30	TTA ATT CCG AAG CTG CAA TCT TCG ATT CAG ACG TAT TAT CCG GGT ACG	1813
	Leu Ile Pro Lys Leu Gln Ser Ser Ile Gln Thr Tyr Tyr Pro Gly Thr	
	365 370 375	
35	AAG CTG GCG ATC ACA GAG TTC AGC TAC GGC GGA GAC AAT CAC ATT TCG	1861
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	380 385 390 395	
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	Gly Gly Ile Ala Thr Ala Asp Ala Leu Gly Ile Phe Gly Lys Tyr Gly	
	400 405 410	
45	GTT TAT GCC GCG AAT TAC TGG CAG ACG GAG GAC AAT ACC GAT TAT ACC	1957
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50	AGC GCT GCT TAC AAG CTG TAT CGC AAC TAC GAC GGC AAT AAA TCG GGG	2005
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55	TTC GGC TCG ATC AAA GTG GAC GCC GCT ACG TCC GAT ACG GAG AAC AGC	2053
	Phe Gly Ser Ile Lys Val Asp Ala Ala Thr Ser Asp Thr Glu Asn Ser	
	445 450 455	
60	TCG GTA TAC GCT TCG GTA ACT GAC GAG GAG AAT TCC GAA CTC CAC CTG	2101
	Ser Val Tyr Ala Ser Val Thr Asp Glu Glu Asn Ser Glu Leu His Leu	
	460 465 470 475	

62

	ATC	GTG	CTG	AAT	AAA	AAT	TTC	GAC	GAT	CCG	ATC	AAC	GCT	ACT	TTC	CAG	2149
	Ile	Val	Leu	Asn	Lys	Asn	Phe	Asp	Asp	Pro	Ile	Asn	Ala	Thr	Phe	Gln	
					480					485					490		
5	CTG	TCT	GGT	GAT	AAA	ACC	TAC	ACA	TCC	GGG	AGA	GTA	TGG	GGC	TTC	GAC	2197
	Leu	Ser	Gly	Asp	Lys	Thr	Tyr	Thr	Ser	Gly	Arg	Val	Trp	Gly	Phe	Asp	
				495					500					505			
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			510					515					520				
15	AAC	AAT	CAA	TTC	ACG	TAT	ACG	CTT	CCT	CCA	TTG	TCG	GCT	TAC	CAC	ATT	2293
	Asn	Asn	Gln	Phe	Thr	Tyr	Thr	Leu	Pro	Pro	Leu	Ser	Ala	Tyr	His	Ile	
			525				530					535					
20	GTT	CTG	AAA	GCG	GAT	AGC	ACC	GAA	CCG	GTC	AAC	TCC	GAT	CTC	GTC	GTG	2341
	Val	Leu	Lys	Ala	Asp	Ser	Thr	Glu	Pro	Val	Asn	Ser	Asp	Leu	Val	Val	
	540				545					550					555		
25	CAG	TAT	AAG	GAC	GGT	GAT	CGC	AAC	AAT	GCA	ACC	GAC	AAT	CAG	ATC	AAG	2389
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				560						565					570		
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				575					580					585			
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			590					595					600				
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	Asn	Gly	Trp	Ile	Asp	Trp	Ala	Lys	Leu	Gly	Gly	Ser	Asn	Ile	Gln	Ile	
		605					610					615					
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						625					630				635		
50	GGC	TTC	TCG	TCC	GGC	GCA	GGC	TCG	ATT	GCG	GAG	GGC	GGT	CAA	TCC	GGC	2629
	Gly	Phe	Ser	Ser	Gly	Ala	Gly	Ser	Ile	Ala	Glu	Gly	Gly	Gln	Ser	Gly	
					640					645					650		
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	Glu	Ile	Gln	Leu	Arg	Met	Ser	Lys	Ala	Asp	Trp	Ser	Asn	Phe	Asn	Glu	
				655					660					665			
60	GCG	AAC	GAC	TAC	TCG	TTC	GAT	GGG	GCG	AAG	ACG	GCC	TAT	ATA	GAT	TGG	2725
	Ala	Asn	Asp	Tyr	Ser	Phe	Asp	Gly	Ala	Lys	Thr	Ala	Tyr	Ile	Asp	Trp	
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63

GAT CGC GTG ACG CTA TAC CAA GAC GGA CAA CTC GTA TGG GGA ATC GAG 2773  
 Asp Arg Val Thr Leu Tyr Gln Asp Gly Gln Leu Val Trp Gly Ile Glu  
 685 690 695

5 CCG TAGAAGATGA CTAGACAACA TTAGTGATGA GACGCGGCCG GCCATAACGG 2826  
 Pro 700

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 10 GCGATTCAAA AGAGAATCGG ATCAATATTG ATGATTGCCT CACTAATTAT TAGCTTATTG 2946  
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15 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 700 amino acids  
 20 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 20 25 30

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 35 35 40 45

Ile Ser Pro Asn Ile Tyr Gly Thr Asn Gln Asp Leu Ser Gly Thr Glu  
 50 55 60

40 Asn Trp Ser Ser Arg Arg Leu Gly Gly Asn Arg Leu Thr Gly Tyr Asn  
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Trp Glu Asn Asn Ala Ser Ser Ala Gly Arg Asp Trp Leu His Tyr Ser  
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45 Asp Asp Phe Leu Cys Gly Asn Gly Gly Val Pro Asp Thr Asp Cys Asp  
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Lys Pro Gly Ala Val Val Thr Ala Phe His Asp Lys Ser Leu Glu Asn  
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50 Gly Ala Tyr Ser Ile Val Thr Leu Gln Met Ala Gly Tyr Val Ser Arg  
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Asp Lys Asn Gly Pro Val Asp Glu Ser Glu Thr Ala Pro Ser Pro Arg  
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 5 Trp Asp Lys Val Glu Phe Ala Lys Asn Ala Pro Phe Ser Leu Gln Pro  
 165 170 175  
 Asp Leu Asn Asp Gly Gln Val Tyr Met Asp Glu Glu Val Asn Phe Leu  
 180 185 190  
 10 Val Asn Arg Tyr Gly Asn Ala Ser Thr Ser Thr Gly Ile Lys Ala Tyr  
 195 200 205  
 Ser Leu Asp Asn Glu Pro Ala Leu Trp Ser Glu Thr His Pro Arg Ile  
 210 215 220  
 15 His Pro Glu Gln Leu Gln Ala Ala Glu Leu Val Ala Lys Ser Ile Asp  
 225 230 235 240  
 20 Leu Ser Lys Ala Val Lys Asn Val Asp Pro His Ala Glu Ile Phe Gly  
 245 250 255  
 Pro Ala Leu Tyr Gly Phe Gly Ala Tyr Leu Ser Leu Gln Asp Ala Pro  
 260 265 270  
 25 Asp Trp Pro Ser Leu Gln Gly Asn Tyr Ser Trp Phe Ile Asp Tyr Tyr  
 275 280 285  
 30 Leu Asp Gln Met Lys Asn Ala His Thr Gln Asn Gly Lys Arg Leu Leu  
 290 295 300  
 Asp Val Leu Asp Val His Trp Tyr Pro Glu Ala Gln Gly Gly Gly Gln  
 305 310 315 320  
 35 Arg Ile Val Phe Gly Gly Ala Gly Asn Ile Asp Thr Gln Lys Ala Arg  
 325 330 335  
 Val Gln Ala Pro Arg Ser Leu Trp Asp Pro Ala Tyr Gln Glu Asp Ser  
 340 345 350  
 40 Trp Ile Gly Thr Trp Phe Ser Ser Tyr Leu Pro Leu Ile Pro Lys Leu  
 355 360 365  
 45 Gln Ser Ser Ile Gln Thr Tyr Tyr Pro Gly Thr Lys Leu Ala Ile Thr  
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 405 410 415

65

Tyr Trp Gln Thr Glu Asp Asn Thr Asp Tyr Thr Ser Ala Ala Tyr Lys  
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 5 Leu Tyr Arg Asn Tyr Asp Gly Asn Lys Ser Gly Phe Gly Ser Ile Lys  
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 10 Val Thr Asp Glu Glu Asn Ser Glu Leu His Leu Ile Val Leu Asn Lys  
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 Asn Phe Asp Asp Pro Ile Asn Ala Thr Phe Gln Leu Ser Gly Asp Lys  
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 15 Thr Tyr Thr Ser Gly Arg Val Trp Gly Phe Asp Gln Thr Gly Ser Asp  
 500 505 510  
 20 Ile Thr Glu Gln Ala Ala Ile Thr Asn Ile Asn Asn Gln Phe Thr  
 515 520 525  
 Tyr Thr Leu Pro Pro Leu Ser Ala Tyr His Ile Val Leu Lys Ala Asp  
 530 535 540  
 25 Ser Thr Glu Pro Val Asn Ser Asp Leu Val Val Gln Tyr Lys Asp Gly  
 545 550 555 560  
 Asp Arg Asn Asn Ala Thr Asp Asn Gln Ile Lys Pro His Phe Asn Ile  
 565 570 575  
 30 Gln Asn Lys Gly Thr Ser Pro Val Asp Leu Ser Ser Leu Thr Leu Arg  
 580 585 590  
 35 Tyr Tyr Phe Thr Lys Asp Ser Ser Ala Ala Met Asn Gly Trp Ile Asp  
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 Trp Ala Lys Leu Gly Gly Ser Asn Ile Gln Ile Ser Phe Gly Asn His  
 610 615 620  
 40 Asn Gly Ala Asp Ser Asp Thr Tyr Ala Glu Leu Gly Phe Ser Ser Gly  
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 45 Met Ser Lys Ala Asp Trp Ser Asn Phe Asn Glu Ala Asn Asp Tyr Ser  
 660 665 670  
 50 Phe Asp Gly Ala Lys Thr Ala Tyr Ile Asp Trp Asp Arg Val Thr Leu  
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66

Tyr Gln Asp Gly Gln Leu Val Trp Gly Ile Glu Pro  
690 695 700

## (2) INFORMATION FOR SEQ ID NO:3:

- 5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2323 base pairs  
 (B) TYPE: nucleic acid  
 10 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 15 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Bacillus lautus*  
 (B) STRAIN: NCIMB 40250
- 20 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 172..1869  
 (D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25 GGGGTTTTGT TCATTCAAG AAAGAGCAG AATAAACATC GCTAATTTAC ACATGATTTA 60  
 TTCTCTAATT ATTATGGTGC ATGCCAATTG GAAATGTAG TAGATTAGTC ATCGTAACCT 120  
 30 GCTTTCATGC CAAGATATGT AATTTTAAA AAAGAGTAAA GGAGAATTCA G ATG AAA 177  
 Met Lys  
 1

35 AAA CGT AGA AGC AGT AAA GTT ATT CTT TCG TTG GCC ATC GTT GTT GCA 225  
 Lys Arg Arg Ser Ser Lys Val Ile Leu Ser Leu Ala Ile Val Val Ala  
 5 10 15

40 TTA TTG GCA GCC GTC GAA CCT AAT GCC GCT TTA GCA GCG GCT CCA CCA 273  
 Leu Leu Ala Ala Val Glu Pro Asn Ala Ala Leu Ala Ala Ala Pro Pro  
 20 25 30

AGT GCC ATG CAG TCC TAT GTT GAA GCG ATG CAG CCT GGC TGG AAC CTT 321  
 Ser Ala Met Gln Ser Tyr Val Glu Ala Met Gln Pro Gly Trp Asn Leu  
 35 40 45 50

45 GGC AAT TCT CTG GAT GCT GTC GGT GCG GAT GAG ACG CTG GCA CGG GGC 369  
 Gly Asn Ser Leu Asp Ala Val Gly Ala Asp Glu Thr Leu Ala Arg Gly  
 55 60 65

50 AAT CCG CGG ATC ACG AAA GAG CTC ATT CAG AAC ATC GCT GCG CAA GGC 417  
 Asn Pro Arg Ile Thr Lys Glu Leu Ile Gln Asn Ile Ala Ala Gln Gly  
 70 75 80



67

	TAT AAG AGC ATA CGG ATT CCT GTT ACC TGG GAT TCC CAT ATC GGC GCG	465
	Tyr Lys Ser Ile Arg Ile Pro Val Thr Trp Asp Ser His Ile Gly Ala	
5	85 90 95	
	GCC CCA AAT TAT CAA ATT GAA GCT GCG TAC CTC AAT CGA GTG CAG GAG	513
	Ala Pro Asn Tyr Gln Ile Glu Ala Ala Tyr Leu Asn Arg Val Gln Glu	
	100 105 110	
10	GTC GTA CAG TGG GCT TTG GAC GCG AAC CTC TAT GTG ATG ATT AAT GTC	561
	Val Val Gln Trp Ala Leu Asp Ala Asn Leu Tyr Val Met Ile Asn Val	
	115 120 125 130	
15	CAT CAT GAT TCC TGG CTA TGG ATC AGC AAA ATG GAG TCG CAG CAC GAT	609
	His His Asp Ser Trp Leu Trp Ile Ser Lys Met Glu Ser Gln His Asp	
	135 140 145	
20	CAA GTA CTG GCC CGT TAT AAT GCG ATT TGG ACG CAA ATT GCC AAC AAG	657
	Gln Val Leu Ala Arg Tyr Asn Ala Ile Trp Thr Gln Ile Ala Asn Lys	
	150 155 160	
25	TTC AAG AAC AGC CCG AGC AAG CTG ATG TTC GAG AGC GTG AAT GAG CCT	705
	Phe Lys Asn Ser Pro Ser Lys Leu Met Phe Glu Ser Val Asn Glu Pro	
	165 170 175	
	CGC TTT ACG GAT GGC GGA ACT ACG GAT GAA GCC AAG CAG CAA AAA ATG	753
	Arg Phe Thr Asp Gly Gly Thr Thr Asp Glu Ala Lys Gln Gln Lys Met	
	180 185 190	
30	CTG GAC GAG CTG AAC GTA TCC TTT TTC AAC ATC GTC AGA AAT TCC GGC	801
	Leu Asp Glu Leu Asn Val Ser Phe Phe Asn Ile Val Arg Asn Ser Gly	
	195 200 205 210	
35	GGC CAG AAC GCG ACT CGC CCG CTA GTT CTT TCT ACG TTG GAG GCC TCT	849
	Gly Gln Asn Ala Thr Arg Pro Leu Val Leu Ser Thr Leu Glu Ala Ser	
	215 220 225	
40	CCC ACC CAA GAG AGA ATG ACG GCG CTT TAT AAT ACG ATG ACC AAA CTG	897
	Pro Thr Gln Glu Arg Met Thr Ala Leu Tyr Asn Thr Met Thr Lys Leu	
	230 235 240	
45	AAC GAC AAG AAT CTG ATC GCA ACC GTT CAT TTT TAT GGA TTC TGG CCG	945
	Asn Asp Lys Asn Leu Ile Ala Thr Val His Phe Tyr Gly Phe Trp Pro	
	245 250 255	
	TTT AGC GTA AAT ATC GCA GGA TAT ACG AAA TTT GAT GCG GAG ACG CAA	993
	Phe Ser Val Asn Ile Ala Gly Tyr Thr Lys Phe Asp Ala Glu Thr Gln	
	260 265 270	
50	AAT GAT ATT ATA ACG ACC TTC GAT AAC GTG TAT AAC ACA TTT GTA GCA	1041
	Asn Asp Ile Ile Thr Thr Phe Asp Asn Val Tyr Asn Thr Phe Val Ala	
	275 280 285 290	

	AAG GGA ATC CCG GTG GTA GTC GGC GAA TAT GGC CTT CTT GGA TTC GAT	1089
	Lys Gly Ile Pro Val Val Val Gly Glu Tyr Gly Leu Leu Gly Phe Asp	
	295 300 305	
5	AAG AAT ACC GGC GTC ATT GAA CAG GGT GAG AAA TTG AAA TTT TTC GAG	1137
	Lys Asn Thr Gly Val Ile Glu Gln Gly Glu Lys Leu Lys Phe Phe Glu	
	310 315 320	
10	TTT TTT GCC CAG TAT GTG AAG CAA AAA AGC ATT TCC ACT ATG CTA TGG	1185
	Phe Phe Ala Gln Tyr Val Lys Gln Lys Ser Ile Ser Thr Met Leu Trp	
	325 330 335	
15	GAT AAC GGA CAG CAC TTC AAC CGC ACG AGC TTC AAG TGG TCT GAC CCG	1233
	Asp Asn Gly Gln His Phe Asn Arg Thr Ser Phe Lys Trp Ser Asp Pro	
	340 345 350	
20	GAT TTA TTC AAT ATG ATC AAG GCC AGT TGG ACC GGA CGT TCA TCC ACG	1281
	Asp Leu Phe Asn Met Ile Lys Ala Ser Trp Thr Gly Arg Ser Ser Thr	
	355 360 365 370	
	GCT TCC AGC GAC CTG ATC CAT GTC AAG CAG GGC ACG GCG GTA AAA GAT	1329
	Ala Ser Ser Asp Leu Ile His Val Lys Gln Gly Thr Ala Val Lys Asp	
	375 380 385	
25	ACT TCG GTT CAG CTC AAT CTT AAC GGG AAT ACG CTA ACT TCC CTT TCC	1377
	Thr Ser Val Gln Leu Asn Leu Asn Gly Asn Thr Leu Thr Ser Leu Ser	
	390 395 400	
30	GTA AAT GGA ACG ACA CTG AAA TCA GGC ACA GAT TAC ACT TTA AAC AGC	1425
	Val Asn Gly Thr Thr Leu Lys Ser Gly Thr Asp Tyr Thr Leu Asn Ser	
	405 410 415	
35	AGC AGA TTA ACT TTT AAA GCG AGC CAG TTG ACC AAG CTG ACC TCC TTG	1473
	Ser Arg Leu Thr Phe Lys Ala Ser Gln Leu Thr Lys Leu Thr Ser Leu	
	420 425 430	
40	GGC AAA TTG GGG GTC AAC GCG ACG ATC GTG ACT AAA TTC AAT AGA GGC	1521
	Gly Lys Leu Gly Val Asn Ala Thr Ile Val Thr Lys Phe Asn Arg Gly	
	435 440 445 450	
	GCC GAC TGG AAG TTC AAC GTA GTC CTG TAC AAT ACG CCT AAG CTT AGC	1569
	Ala Asp Trp Lys Phe Asn Val Val Leu Tyr Asn Thr Pro Lys Leu Ser	
	455 460 465	
45	AGT ACG ACG GGG ACT ACT TCT TCC TTT GCG ATT CCA ACG GCT TTC AAC	1617
	Ser Thr Thr Gly Thr Thr Ser Ser Phe Ala Ile Pro Thr Ala Phe Asn	
	470 475 480	
50	GGG GAT CAG CTT GCT ACG ATG GAA GCG GTC TAT GTA AAC GGC GGC AAT	1665
	Gly Asp Gln Leu Ala Thr Met Glu Ala Val Tyr Val Asn Gly Gly Asn	
	485 490 495	

69

	GCC GGT CCG CAT AAC TGG ACT TCC TTT AAG GAA TTC GAA ACG ACG TTC	1713
	Ala Gly Pro His Asn Trp Thr Ser Phe Lys Glu Phe Glu Thr Thr Phe	
5	500 505 510	
	AGC CCC GCT TAT AGC GAG GGG AAA ATC AAA CTG CAG CAG GCG TTC TTT	1761
	Ser Pro Ala Tyr Ser Glu Gly Lys Ile Lys Leu Gln Gln Ala Phe Phe	
	515 520 525 530	
10	AAT GAA GTG AAT GAT ACC ACA GTC ACG CTC AAG TTC CAA TTC TGG AGC	1809
	Asn Glu Val Asn Asp Thr Thr Val Thr Leu Lys Phe Gln Phe Trp Ser	
	535 540 545	
15	GGG GAG ATC GTC AAC TAC ACG ATT AAA AAG AGC GGT TCG ACG GTG ACG	1857
	Gly Glu Ile Val Asn Tyr Thr Ile Lys Lys Ser Gly Ser Thr Val Thr	
	550 555 560	
20	GGT ACG GCT TCA TAAGCGAGTT TGGCAAAAAA GGACCGATAT ACTGCCTAAT	1909
	Gly Thr Ala Ser	
	565	
	TTGGTATTGC CTTAGTTGAA AGCAATTGCT CCGAATAAAC AGAATGAAGC CCCGGCCAGC	1969
25	TGGCCGGGAC TTATGCGTTT AGGAAGTATA AACGAATCAT CAGCAATTTA TTTAGCTCGT	2029
	CTCAGTTCAG CAATATCGGC TTCATGTGAA ACGGAGCGGA TGAACAATCT TTCGAGCAAT	2089
	TTCTCATGCT CCTGCTGGGT TTGGAGAACG GTTTGCTGAT TAGTTTTAAG TACAGATATA	2149
30	TCCTCACGGA CTTGATTGAT TCATGTGGTC CGTTAGTTCT TCTACCTTTG TATTGTGGC	2209
	AGCAACGATA TGAATTAATT GTTGAATGTG CCCGCCATGA CTGTTTAGCT GCTCATTGTG	2269
35	GCTTTGTAAC TGTTCGGA TTTCTTTGAA TTCTTGGTCG TGCTCATTAA GCTT	2323

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 566 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Lys Arg Arg Ser Ser Lys Val Ile Leu Ser Leu Ala Ile Val  
1 5 10 15  
50 Val Ala Leu Leu Ala Ala Val Glu Pro Asn Ala Ala Leu Ala Ala  
20 25 30

70

Pro Pro Ser Ala Met Gln Ser Tyr Val Glu Ala Met Gln Pro Gly Trp  
                   35                                  40                                  45  
 5 Asn Leu Gly Asn Ser Leu Asp Ala Val Gly Ala Asp Glu Thr Leu Ala  
           50                                  55                                  60  
 Arg Gly Asn Pro Arg Ile Thr Lys Glu Leu Ile Gln Asn Ile Ala Ala  
   65                                  70                                  75                                  80  
 10 Gln Gly Tyr Lys Ser Ile Arg Ile Pro Val Thr Trp Asp Ser His Ile  
                                   85                                  90                                  95  
 Gly Ala Ala Pro Asn Tyr Gln Ile Glu Ala Ala Tyr Leu Asn Arg Val  
   15                                  100                                  105                                  110  
 Gln Glu Val Val Gln Trp Ala Leu Asp Ala Asn Leu Tyr Val Met Ile  
                                   115                                  120                                  125  
 20 Asn Val His His Asp Ser Trp Leu Trp Ile Ser Lys Met Glu Ser Gln  
           130                                  135                                  140  
 His Asp Gln Val Leu Ala Arg Tyr Asn Ala Ile Trp Thr Gln Ile Ala  
   145                                  150                                  155                                  160  
 25 Asn Lys Phe Lys Asn Ser Pro Ser Lys Leu Met Phe Glu Ser Val Asn  
                                   165                                  170                                  175  
 Glu Pro Arg Phe Thr Asp Gly Gly Thr Thr Asp Glu Ala Lys Gln Gln  
   30                                  180                                  185                                  190  
 Lys Met Leu Asp Glu Leu Asn Val Ser Phe Phe Asn Ile Val Arg Asn  
                                   195                                  200                                  205  
 35 Ser Gly Gly Gln Asn Ala Thr Arg Pro Leu Val Leu Ser Thr Leu Glu  
           210                                  215                                  220  
 Ala Ser Pro Thr Gln Glu Arg Met Thr Ala Leu Tyr Asn Thr Met Thr  
   225                                  230                                  235                                  240  
 40 Lys Leu Asn Asp Lys Asn Leu Ile Ala Thr Val His Phe Tyr Gly Phe  
                                   245                                  250                                  255  
 Trp Pro Phe Ser Val Asn Ile Ala Gly Tyr Thr Lys Phe Asp Ala Glu  
   45                                  260                                  265                                  270  
 Thr Gln Asn Asp Ile Ile Thr Thr Phe Asp Asn Val Tyr Asn Thr Phe  
                                   275                                  280                                  285  
 50 Val Ala Lys Gly Ile Pro Val Val Val Gly Glu Tyr Gly Leu Leu Gly  
           290                                  295                                  300

71

Phe Asp Lys Asn Thr Gly Val Ile Glu Gln Gly Glu Lys Leu Lys Phe  
 305 310 315 320  
 Phe Glu Phe Phe Ala Gln Tyr Val Lys Gln Lys Ser Ile Ser Thr Met  
 5 325 330 335  
 Leu Trp Asp Asn Gly Gln His Phe Asn Arg Thr Ser Phe Lys Trp Ser  
 340 345 350  
 10 Asp Pro Asp Leu Phe Asn Met Ile Lys Ala Ser Trp Thr Gly Arg Ser  
 355 360 365  
 Ser Thr Ala Ser Ser Asp Leu Ile His Val Lys Gln Gly Thr Ala Val  
 15 370 375 380  
 Lys Asp Thr Ser Val Gln Leu Asn Leu Asn Gly Asn Thr Leu Thr Ser  
 385 390 395 400  
 20 Leu Ser Val Asn Gly Thr Thr Leu Lys Ser Gly Thr Asp Tyr Thr Leu  
 405 410 415  
 Asn Ser Ser Arg Leu Thr Phe Lys Ala Ser Gln Leu Thr Lys Leu Thr  
 420 425 430  
 25 Ser Leu Gly Lys Leu Gly Val Asn Ala Thr Ile Val Thr Lys Phe Asn  
 435 440 445  
 Arg Gly Ala Asp Trp Lys Phe Asn Val Val Leu Tyr Asn Thr Pro Lys  
 30 450 455 460  
 Leu Ser Ser Thr Thr Gly Thr Thr Ser Ser Phe Ala Ile Pro Thr Ala  
 465 470 475 480  
 35 Phe Asn Gly Asp Gln Leu Ala Thr Met Glu Ala Val Tyr Val Asn Gly  
 485 490 495  
 Gly Asn Ala Gly Pro His Asn Trp Thr Ser Phe Lys Glu Phe Glu Thr  
 500 505 510  
 40 Thr Phe Ser Pro Ala Tyr Ser Glu Gly Lys Ile Lys Leu Gln Gln Ala  
 515 520 525  
 Phe Phe Asn Glu Val Asn Asp Thr Thr Val Thr Leu Lys Phe Gln Phe  
 45 530 535 540  
 Trp Ser Gly Glu Ile Val Asn Tyr Thr Ile Lys Lys Ser Gly Ser Thr  
 545 550 555 560  
 50 Val Thr Gly Thr Ala Ser  
 565

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1775 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

10

## (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus loutus*  
 (B) STRAIN: NCIMB 40250

15

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 30..(1625.1775)  
 (C) IDENTIFICATION METHOD: experimental  
 (D) OTHER INFORMATION: /partial  
 /evidence= EXPERIMENTAL  
 /transl\_except= (pos: 1446 .. 1458, aa: OTR)

20

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

25 TTGAAGCGCT GAATTCAGGA GGTAAATAA TCGTATTCA TGCAATTCGG CAATCTTGCC 60  
 30 GTTTGGTATT GACGATGGTT TTGATGCTTG GCTTATTGCT GCCTGTGGGC GCCCCAAAG 120  
 GCTATGCCGC TCCGGCTGTT CCTTTTGGCC AATTAAAAGT TCAGGGCAAT CAATTGGTAG 180  
 35 GACAGTCCGG GCAAGCTGTT CAACTGGTTG GCATGAGCTC ACATGGATTG CAGTGGTATG 240  
 GCAATTTCTG CAACAAATCG TCGTTGCAGT GGATGAGAGA TAACTGGGGC ATCAACGTCT 300  
 TCCGTGCCGC TATGTATACT TCCGAAGATG GTTACATTAC GGATCCTTCC GTCAAGAACA 360  
 40 AGGTGAAGGA GCGGGTTCAG GCATCCATCG ATCTGGCCTT GTACGTTATT ATTGACTGGC 420  
 ATATCTTGTC TGATGGGAAT CCGAATACGT ACAAAGCGCA ATCGAAAGCG TTCTTCCAAG 480  
 45 AGATGGCCAC ATTGTACGGC AACACGCCGA ATGTAATCTA TGAAATCGCG ACGAGCCCAA 540  
 CGGAATGTGT CCTGGGCAGA TGTCAGTCGT CGGAAGAAGT GATCACGGCC ATTCGTTCTGA 600  
 TTGACCCCGA CGGAGTGGTT ATCGTTGGCA GTCCAACCTG GAGCCAGGAT ATTCATCTGG 660  
 50 CGGCCGATAA CCCGGTATCA CATAGCAATG TCATGTATGC GCTTCATTC TATTCAGGCA 720  
 CGCATGGACA GTTTTTGAGA GACCGAATTA CCTATGCGAT GAACAAAGGA GCAGCGATCT 780

TCGTTACCGA GTGGGGCACC AGTGATGCAT CCGGGAACGG CGGGCCGTAT TTGCCTCAGT 840  
CCAAAGAGTG GATCGATTTC TTGAATGCTC GCAAGATCAG CTGGGTGAAC TGGTCGCTCG 900  
5 CTGATAAAGT AGAAACGTCT GCTGCTCTTA TGCCAGGTGC ATCGCCTACC GGCCTGGAC 960  
CGATGCCCAA TTGTGCAATG GGCAAATCGG GTTCGCGATC AAATCCGGCA AGCAACTGGA 1020  
10 GGC GGCGAGGG CAATCCAAC TGCACGGCTG CCCCTACTAA CCTCTCGGCA AACGGCGGCA 1080  
ACGCCCAGGT ATCATTAAAC TGGAACGCAG TTAGCGGGG GACGAGCTAT ACCGTAAAGC 1140  
GAGCAACGAC GAGCGGCGGT CCGTACACGA ATGTGGACCG GGGTGTACG GCGACGAGCT 1200  
15 ACACGAACAC CGGGCTGACG AATGGCACGA CGTATTATTA TGTCGTGAGG GCATCCAATA 1260  
GCGCGGGCAG CAGCGCGAAC TCCGCGCAAG CGAGCGCAAC GCCGGCTAGC GGC GGCGGCCA 1320  
20 GTACGGGGAA CCTTGTTGTC CAATACAAAG TTGGCGACAC TAGCGCCACG GATAACCAAA 1380  
TGAAGCCTTC CTTTAACATC AAGAACAACG GTACAACCCC TGTTAACCTG AGCGGCCTCA 1440  
AGCTTNNNNN NNNNNNNNAA AAAGACGGAC CTGCGGATAT GAGCTGCTCG ATCGACTGGG 1500  
25 CGCAAATCGG CCGAACGAAT GTTCTGCTGG CATTGCTAA CTTTACCGGG AGTAATACGG 1560  
ATACTTACTG TTGTGAGCTA AGCTTTAGCT GCACTGCAGG TTCGTATCCC GGCTATGCGT 1620  
30 GGACNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 1680  
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 1740  
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNN 1775

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 1609 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

45

## (v) FRAGMENT TYPE: N-terminal

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus lautus*  
50 (B) STRAIN: NCIMB 40250

74

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 30..1607

(D) OTHER INFORMATION:

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10	TTGAAGCGCT GAATTCAGGA GGTAAATA ATG CGT ATT CAT GCA ATT CGG CAA	53
	Met Arg Ile His Ala Ile Arg Gln	
	1 5	
15	TCT TGC CGT TTG GTA TTG ACG ATG GTT TTG ATG CTT GGC TTA TTG CTG	101
	Ser Cys Arg Leu Val Leu Thr Met Val Leu Met Leu Gly Leu Leu Leu	
	10 15 20	
20	CCT GTG GGC GCC CCC AAA GGC TAT GCC GCT CCG GCT GTT CCT TTT GGC	149
	Pro Val Gly Ala Pro Lys Gly Tyr Ala Ala Pro Ala Val Pro Phe Gly	
	25 30 35 40	
25	CAA TTA AAA GTT CAG GGC AAT CAA TTG GTA GGA CAG TCC GGG CAA GCT	197
	Gln Leu Lys Val Gln Gly Asn Gln Leu Val Gly Gln Ser Gly Gln Ala	
	45 50 55	
30	GTT CAA CTG GTT GGC ATG AGC TCA CAT GGA TTG CAG TGG TAT GGC AAT	245
	Val Gln Leu Val Gly Met Ser Ser His Gly Leu Gln Trp Tyr Gly Asn	
	60 65 70	
35	TTC GTC AAC AAA TCG TCG TTG CAG TGG ATG AGA GAT AAC TGG GGC ATC	293
	Phe Val Asn Lys Ser Ser Leu Gln Trp Met Arg Asp Asn Trp Gly Ile	
	75 80 85	
40	AAC GTC TTC CGT GCC GCT ATG TAT ACT TCC GAA GAT GGT TAC ATT ACG	341
	Asn Val Phe Arg Ala Ala Met Tyr Thr Ser Glu Asp Gly Tyr Ile Thr	
	90 95 100	
45	GAT CCT TCC GTC AAG AAC AAG GTG AAG GAG GCG GTT CAG GCA TCC ATC	389
	Asp Pro Ser Val Lys Asn Lys Val Lys Glu Ala Val Gln Ala Ser Ile	
	105 110 115 120	
50	GAT CTG GCC TTG TAC GTT ATT ATT GAC TGG CAT ATC TTG TCT GAT GGG	437
	Asp Leu Ala Leu Tyr Val Ile Ile Asp Trp His Ile Leu Ser Asp Gly	
	125 130 135	
55	AAT CCG AAT ACG TAC AAA GCG CAA TCG AAA GCG TTC TTC CAA GAG ATG	485
	Asn Pro Asn Thr Tyr Lys Ala Gln Ser Lys Ala Phe Phe Gln Glu Met	
	140 145 150	
60	GCC ACA TTG TAC GGC AAC ACG CCG AAT GTA ATC TAT GAA ATC GCG ACG	533
	Ala Thr Leu Tyr Gly Asn Thr Pro Asn Val Ile Tyr Glu Ile Ala Thr	
	155 160 165	



75

	AGC CCA ACG GAA TGT GTC CTG GGC AGA TGT CAG TCG TCG GAA GAA GTG	581
	Ser Pro Thr Glu Cys Val Leu Gly Arg Cys Gln Ser Ser Glu Glu Val	
	170 175 180	
5	ATC ACG GCC ATT CGT TCG ATT GAC CCC GAC GGA GTG GTT ATC GTT GGC	629
	Ile Thr Ala Ile Arg Ser Ile Asp Pro Asp Gly Val Val Ile Val Gly	
	185 190 195 200	
10	AGT CCA ACC TGG AGC CAG GAT ATT CAT CTG GCG GCC GAT AAC CCG GTA	677
	Ser Pro Thr Trp Ser Gln Asp Ile His Leu Ala Ala Asp Asn Pro Val	
	205 210 215	
15	TCA CAT AGC AAT GTC ATG TAT GCG CTT CAT TTC TAT TCA GGC ACG CAT	725
	Ser His Ser Asn Val Met Tyr Ala Leu His Phe Tyr Ser Gly Thr His	
	220 225 230	
20	GGA CAG TTT TTG AGA GAC CGA ATT ACC TAT GCG ATG AAC AAA GGA GCA	773
	Gly Gln Phe Leu Arg Asp Arg Ile Thr Tyr Ala Met Asn Lys Gly Ala	
	235 240 245	
25	GCG ATC TTC GTT ACC GAG TGG GGC ACC AGT GAT GCA TCC GGG AAC GGC	821
	Ala Ile Phe Val Thr Glu Trp Gly Thr Ser Asp Ala Ser Gly Asn Gly	
	250 255 260	
30	GGG CCG TAT TTG CCT CAG TCC AAA GAG TGG ATC GAT TTC TTG AAT GCT	869
	Gly Pro Tyr Leu Pro Gln Ser Lys Glu Trp Ile Asp Phe Leu Asn Ala	
	265 270 275 280	
35	CGC AAG ATC AGC TGG GTG AAC TGG TCG CTC GCT GAT AAA GTA GAA ACG	917
	Arg Lys Ile Ser Trp Val Asn Trp Ser Leu Ala Asp Lys Val Glu Thr	
	285 290 295	
40	TCT GCT GCT CTT ATG CCA GGT GCA TCG CCT ACC GGC GCT GGA CCG ATG	965
	Ser Ala Ala Leu Met Pro Gly Ala Ser Pro Thr Gly Ala Gly Pro Met	
	300 305 310	
45	CCC AAT TGT CGA ATG GGC AAA TCG GGT TCG CGA TCA AAT CCG GCA AGC	1013
	Pro Asn Cys Arg Met Gly Lys Ser Gly Ser Arg Ser Asn Pro Ala Ser	
	315 320 325	
50	AAC TGG AGG CGG CAG GGC AAT CCA ACT GCA CCG GCT GCC CCT ACT AAC	1061
	Asn Trp Arg Arg Gln Gly Asn Pro Thr Ala Pro Ala Ala Pro Thr Asn	
	330 335 340	
55	CTC TCG GCA AAC GGC GGC AAC GCC CAG GTA TCA TTA ACC TGG AAC GCA	1109
	Leu Ser Ala Asn Gly Gly Asn Ala Gln Val Ser Leu Thr Trp Asn Ala	
	345 350 355 360	
60	GTT AGC GGG GCG ACG AGC TAT ACC GTA AAG CGA GCA ACG ACG AGC GGC	1157
	Val Ser Gly Ala Thr Ser Tyr Thr Val Lys Arg Ala Thr Thr Ser Gly	
	365 370 375	

76

	GGT CCG TAC ACG AAT GTG GAC CGG GGT GTC ACG GCG ACG AGC TAC ACG	1205
	Gly Pro Tyr Thr Asn Val Asp Arg Gly Val Thr Ala Thr Ser Tyr Thr	
	380 385 390	
5	AAC ACC GGG CTG ACG AAT GGC ACG ACG TAT TAT TAT GTC GTG AGG GCA	1253
	Asn Thr Gly Leu Thr Asn Gly Thr Thr Tyr Tyr Tyr Val Val Arg Ala	
	395 400 405	
10	TCC AAT AGC GCG GGC AGC AGC GCG AAC TCC GCG CAA GCG AGC GCA ACG	1301
	Ser Asn Ser Ala Gly Ser Ser Ala Asn Ser Ala Gln Ala Ser Ala Thr	
	410 415 420	
15	CCG GCT AGC GGC GGC GCC AGT ACG GGG AAC CTT GTT GTC CAA TAC AAA	1349
	Pro Ala Ser Gly Gly Ala Ser Thr Gly Asn Leu Val Val Gln Tyr Lys	
	425 430 435 440	
20	GTT GGC GAC ACT AGC GCC ACG GAT AAC CAA ATG AAG CCT TCC TTT AAC	1397
	Val Gly Asp Thr Ser Ala Thr Asp Asn Gln Met Lys Pro Ser Phe Asn	
	445 450 455	
25	ATC AAG AAC AAC GGT ACA ACC CCT GTT AAC CTG AGC GGC CTC AAG CTT	1445
	Ile Lys Asn Asn Gly Thr Thr Pro Val Asn Leu Ser Gly Leu Lys Leu	
	460 465 470	
30	NNN NNN NNN NNN NAA AAA GAC GGA CCT GCG GAT ATG AGC TGC TCG ATC	1493
	Xaa Xaa Xaa Xaa Xaa Lys Asp Gly Pro Ala Asp Met Ser Cys Ser Ile	
	475 480 485	
35	GAC TGG GCG CAA ATC GGC CGA ACG AAT GTT CTG CTG GCA TTC GCT AAC	1541
	Asp Trp Ala Gln Ile Gly Arg Thr Asn Val Leu Leu Ala Phe Ala Asn	
	490 495 500	
40	TTT ACC GGG AGT AAT ACG GAT ACT TAC TGT TGT GAG CTA AGC TTT AGC	1589
	Phe Thr Gly Ser Asn Thr Asp Thr Tyr Cys Cys Glu Leu Ser Phe Ser	
	505 510 515 520	
45	TGC ACT GCA GGT TCG TAT CCC GGC TAT GCG TGG AC	1624
	Cys Thr Ala Gly Ser Tyr Pro Gly Tyr Ala Trp	
	525	

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 526 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

77

Met Arg Ile His Ala Ile Arg Gln Ser Cys Arg Leu Val Leu Thr Met  
 1 5 10 15  
 Val Leu Met Leu Gly Leu Leu Leu Pro Val Gly Ala Pro Lys Gly Tyr  
 5 20 25 30  
 Ala Ala Pro Ala Val Pro Phe Gly Gln Leu Lys Val Gln Gly Asn Gln  
 35 40 45  
 10 Leu Val Gly Gln Ser Gly Gln Ala Val Gln Leu Val Gly Met Ser Ser  
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 His Gly Leu Gln Trp Tyr Gly Asn Phe Val Asn Lys Ser Ser Leu Gln  
 65 70 75 80  
 15 Trp Met Arg Asp Asn Trp Gly Ile Asn Val Phe Arg Ala Ala Met Tyr  
 85 90 95  
 Thr Ser Glu Asp Gly Tyr Ile Thr Asp Pro Ser Val Lys Asn Lys Val  
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 Lys Glu Ala Val Gln Ala Ser Ile Asp Leu Ala Leu Tyr Val Ile Ile  
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 25 Asp Trp His Ile Leu Ser Asp Gly Asn Pro Asn Thr Tyr Lys Ala Gln  
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 Ser Lys Ala Phe Phe Gln Glu Met Ala Thr Leu Tyr Gly Asn Thr Pro  
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 40 His Leu Ala Ala Asp Asn Pro Val Ser His Ser Asn Val Met Tyr Ala  
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 Leu His Phe Tyr Ser Gly Thr His Gly Gln Phe Leu Arg Asp Arg Ile  
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 45 Thr Tyr Ala Met Asn Lys Gly Ala Ala Ile Phe Val Thr Glu Trp Gly  
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 Thr Ser Asp Ala Ser Gly Asn Gly Gly Pro Tyr Leu Pro Gln Ser Lys  
 50 260 265 270

78

Glu Trp Ile Asp Phe Leu Asn Ala Arg Lys Ile Ser Trp Val Asn Trp  
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 500 505 510  
 45 Tyr Cys Cys Glu Leu Ser Phe Ser Cys Thr Ala Gly Ser Tyr Pro Gly  
 515 520 525  
 50 Tyr Ala Trp  
 530

Optional Sheet in connection with the microorganism referred to on page 3, line 20 of the description:

Further deposits are identified on an additional sheet ☐:

NATIONAL COLLECTION OF INDUSTRIAL & MARINE BACTERIA  
LTD.

**Torry Research Station, P.O. Box 31, 23 St Machar Drive, Aberdeen AB9 8DG, Scotland**

18 January 1990

NCIMB 40250

In respect of those designations in which a European patent is sought, a sample of the deposited micro-organism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

**D. SEPARATE FURNISHING OF INDICATIONS:** *Circle Mark if not applicable*

The indications listed below will be submitted to the International Bureau later. (Specify the general nature of the indications a.s., "Accession Number of Deposit")

8. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Clyonne Jakobsen  
(Authorized Officer)      Muzanne Jakobsen

**Yvonne Jakobson:**  
Head Clerk

☐ The date of receipt (from the applicant) by the International Bureau is :

534

(Authorized Officer)

## CLAIMS

1. An enzyme which exhibits cellulase activity, which enzyme is producible by a strain of Bacillus spp., NCIMB 40250, or a related Bacillus spp. strain,  
5 or a derivative of said cellulase.
2. An enzyme according to claim 1, which exhibits an endoglucanase activity of at least about 10, more preferably at least about 20, most preferably at least about 25, such as about 30, CMC-endoase units (as defined herein) per  
10 mg of total protein under alkaline conditions.
3. An enzyme according to claim 1, which is active at a temperature of up to about 65 °C.
- 15 4. An enzyme according to any of claims 1-3, which is an endoglucanase with an apparent molecular weight of 75 kD or a cleavage product thereof exhibiting endoglucanase activity.
5. An enzyme according to claim 4, which is an endoglucanase encoded by the  
20 DNA sequence shown in the appended Sequence Listing ID#1, or a modification thereof encoding a derivative of said endoglucanase.
6. An enzyme according to any of claims 1-3, which is an endoglucanase with an apparent molecular weight of 56 kD or a cleavage product thereof  
25 exhibiting endoglucanase activity.
7. An enzyme according to claim 6, which is an endoglucanase encoded by the DNA sequence shown in the appended Sequence Listing ID##, or a modification thereof encoding a derivative of said endoglucanase.  
30
8. An enzyme according to any of claims 1-3, which is an endoglucanase with an apparent molecular weight of 45 kD or a cleavage product thereof exhibiting endoglucanase activity.

9. An enzyme according to claim 8, which is an endoglucanase encoded by the DNA sequence shown in the appended Sequence Listing ID#6, or a modification thereof encoding a derivative of said endoglucanase.
- 5 10. An enzyme according to any of claims 1-3, which is an endoglucanase with an apparent molecular weight of 60 kD or a cleavage product thereof exhibiting endoglucanase activity, or which is an endoglucanase with an apparent molecular weight of 56 kD or a cleavage product thereof exhibiting endoglucanase activity.
- 10 11. An enzyme according to any of claims 1-3, which is an endoglucanase with an apparent molecular weight of 92 kD or a cleavage product thereof exhibiting endoglucanase activity.
- 15 12. An enzyme which comprises a core region derived from an endoglucanase according to any of claims 1-11 combined with a cellulose-binding domain derived from another cellulase enzyme, or a core region derived from another cellulase enzyme combined with a cellulose-binding domain derived from an endoglucanase according to any of claims 1-11.
- 20 13. An enzyme according to claim 12, wherein the core region is derived from a cellulase enzyme which does not, in nature, comprise a cellulose-binding domain.
- 25 14. A DNA construct which comprises a DNA sequence encoding an enzyme exhibiting cellulase activity, which enzyme is derivable from a strain of Bacillus spp., NCIMB 40250, or a related Bacillus spp. strain, or a derivative of said cellulase.
- 30 15. A DNA construct according to claim 14, wherein the DNA sequence encodes an endoglucanase with an apparent molecular weight of 75 kD.
- 35 16. A DNA construct according to claim 15, wherein the DNA sequence is the one shown in the appended Sequence Listing ID#1, or a modification thereof encoding a derivative of said endoglucanase.

17. A DNA construct according to claim 14, wherein the DNA sequence encodes an endoglucanase with an apparent molecular weight of 56 kD.
- 5 18. A DNA construct according to claim 17, wherein the DNA sequence is the one shown in the appended Sequence Listing ID#3, or a modification thereof encoding a derivative of said endoglucanase.
19. A DNA construct according to claim 14, wherein the DNA sequence encodes an endoglucanase with an apparent molecular weight of 45 kD.
- 10 20. A DNA construct according to claim 19, wherein the DNA sequence is the one shown in the appended Sequence Listing ID#6, or a modification thereof encoding a derivative of said endoglucanase.
- 15 21. A DNA construct according to claim 14, wherein the DNA sequence encodes an endoglucanase with an apparent molecular weight of 92 kD.
22. An expression vector which carries an inserted DNA construct according to any of claims 14-21.
- 20 23. A cell which is transformed with a DNA construct according to any of claims 14-21 or with an expression vector according to claim 22.
- 25 24. A cell according to claim 23, which is a bacterium.
25. A cell according to claim 24, which is a grampositive bacterium.
26. A cell according to claim 25, wherein the grampositive bacterium is selected from the group consisting of Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans or Bacillus lautus.
- 30



27. A cell according to claim 24, which is a gramnegative bacterium, e.g. Escherichia coli.
28. A method of producing an enzyme according to any of claims 1-13, wherein  
5 a host cell according to any of claims 23-27 is cultured under conditions conducive to the production of the endoglucanase or a derivative thereof, and the endoglucanase or derivative thereof is subsequently recovered from the culture.
29. A method according to claim 28, wherein the endoglucanase is recovered  
10 in mature form.
30. A cellulolytic agent capable of degrading amorphous regions of cellulose  
15 fibres, the agent comprising an enzyme according to any of claims 1-13.
31. An agent according to claim 30, which comprises a combination of two or more cellulases recited in any of claims 1-13, or a combination of one or more cellulases recited in any of claims 1-13 with one or more other enzymes with cellulase activity.
32. An agent according to claim 30 or 31, which is in the form of a non-dusting granulate, stabilized liquid or protected enzyme.
33. An agent according to any of claims 30-32, which exhibits an  
25 endoglucanase activity of 500-10,000 CMC-endoase units per gram of the agent.
34. An agent according to any of claims 30-33, which is a detergent additive.
35. An agent according to claim 34, which additionally comprises another  
30 enzyme such as a protease, lipase and/or amylase.
36. A detergent composition comprising a cellulolytic agent according to any of claims 30-35.

37. A detergent composition according to claim 36, which exhibits an endoglucanase activity of 0.3-400 CMC-endoase units per gram of detergent.
38. A method of reducing the rate at which cellulose-containing fabrics become harsh or of reducing the harshness of cellulose-containing fabrics, the method comprising treating cellulose-containing fabrics with a cellulolytic agent according to any of claims 30-35.
39. A method according to claim 38, wherein the treatment of the fabrics with the cellulolytic agent is conducted during soaking, washing or rinsing of the fabrics.
40. A method of treating a coloured, cellulose-containing fabric in order to provide colour clarification, the method comprising treating the cellulose-containing fabric with a cellulolytic agent according to any of claims 30-35.
41. A method according to claim 40, wherein the treatment of the fabric with the cellulolytic agent is conducted in an aqueous medium during soaking, washing or rinsing of the fabric.
42. A method according to claim 41, wherein the aqueous medium exhibits an endoglucanase activity of more than about 250 CMC-endoase units per liter of the aqueous medium.

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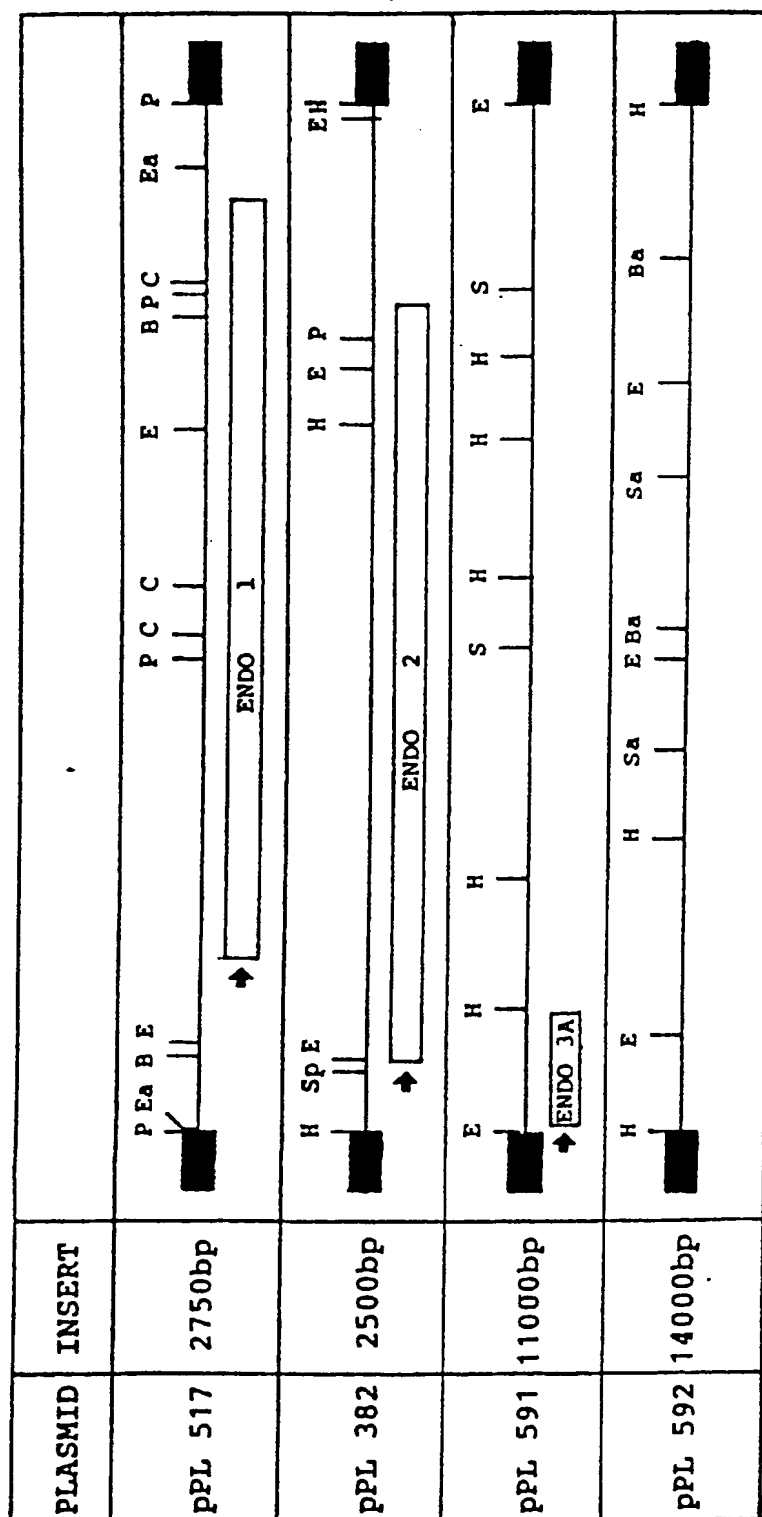


Fig. 1

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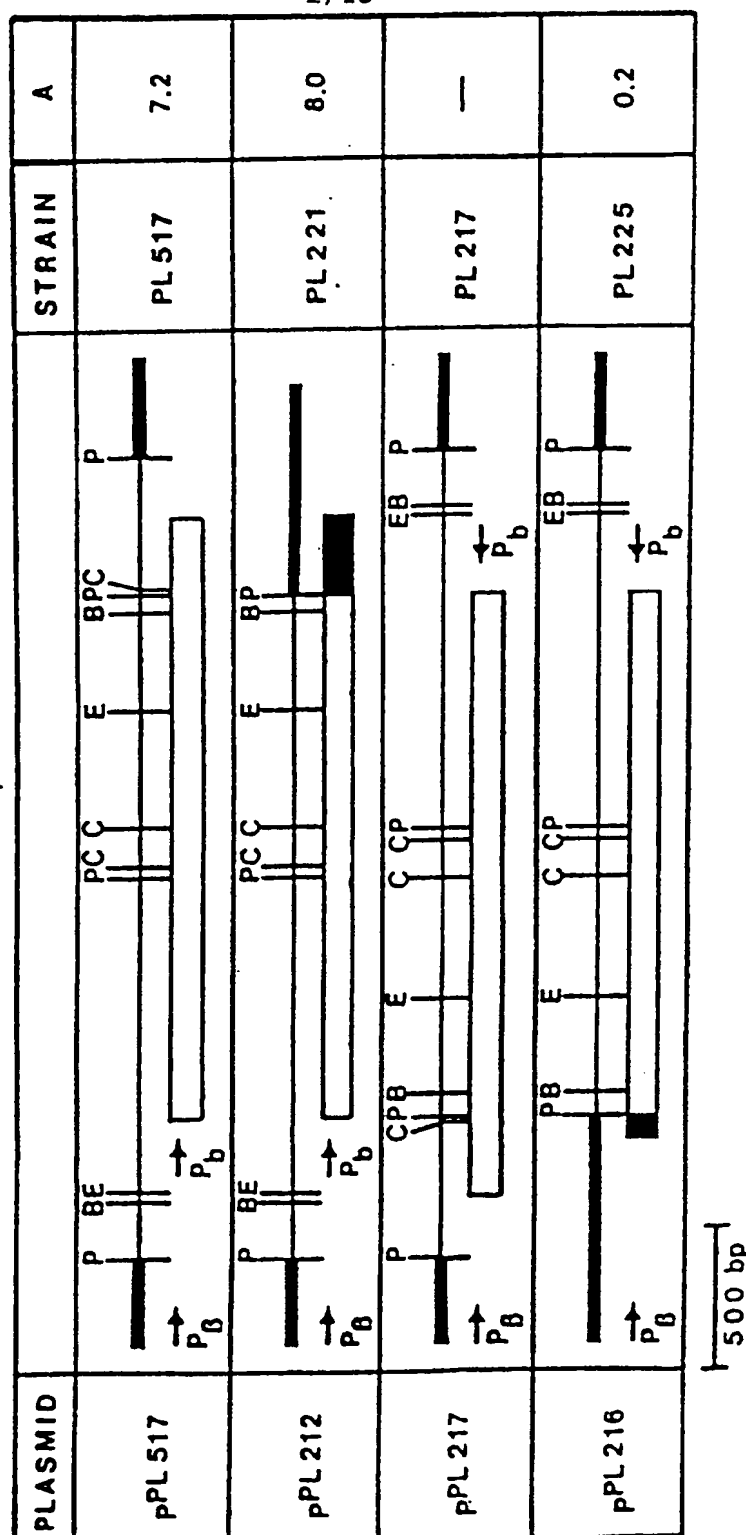


Fig. 2

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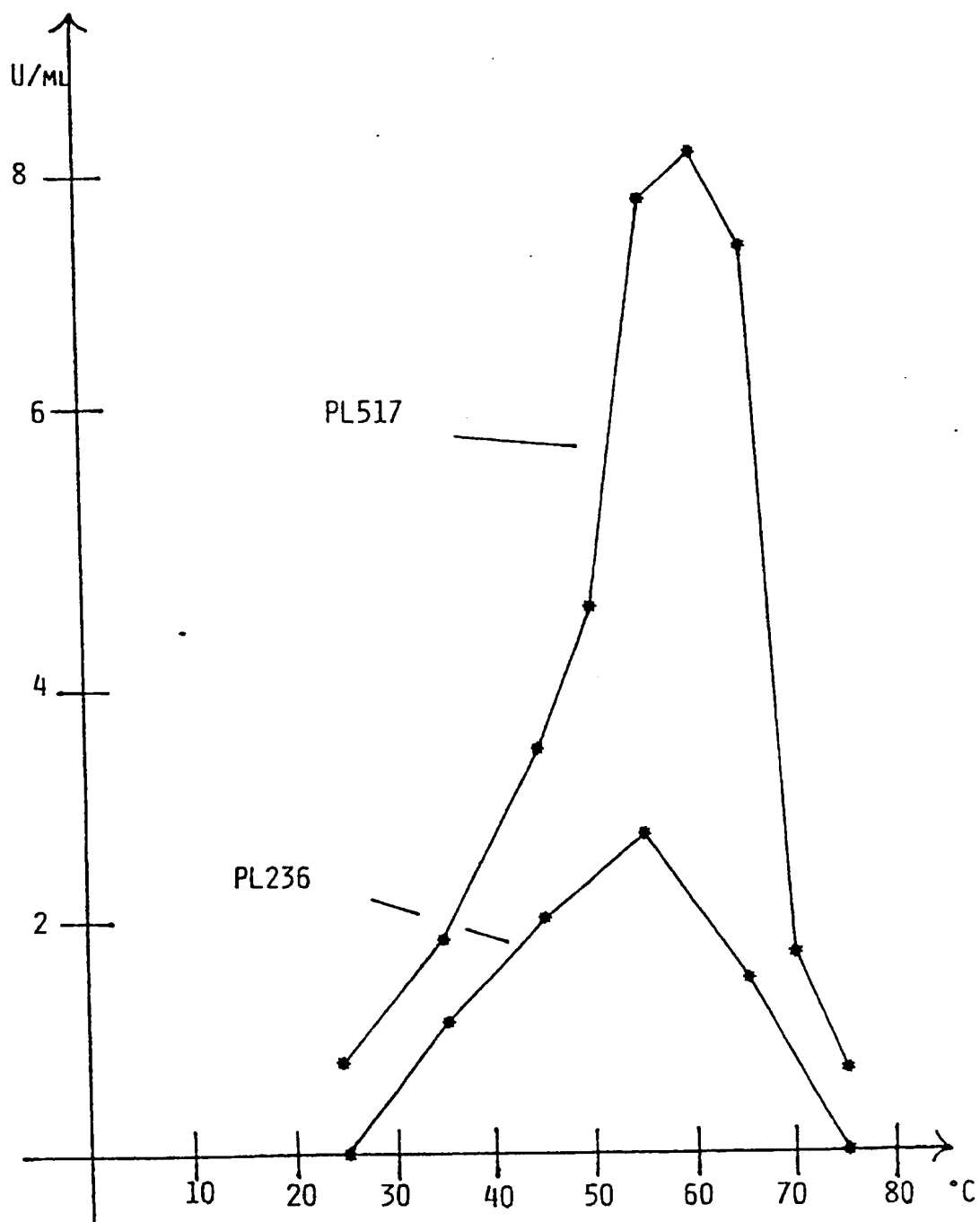


Fig. 3

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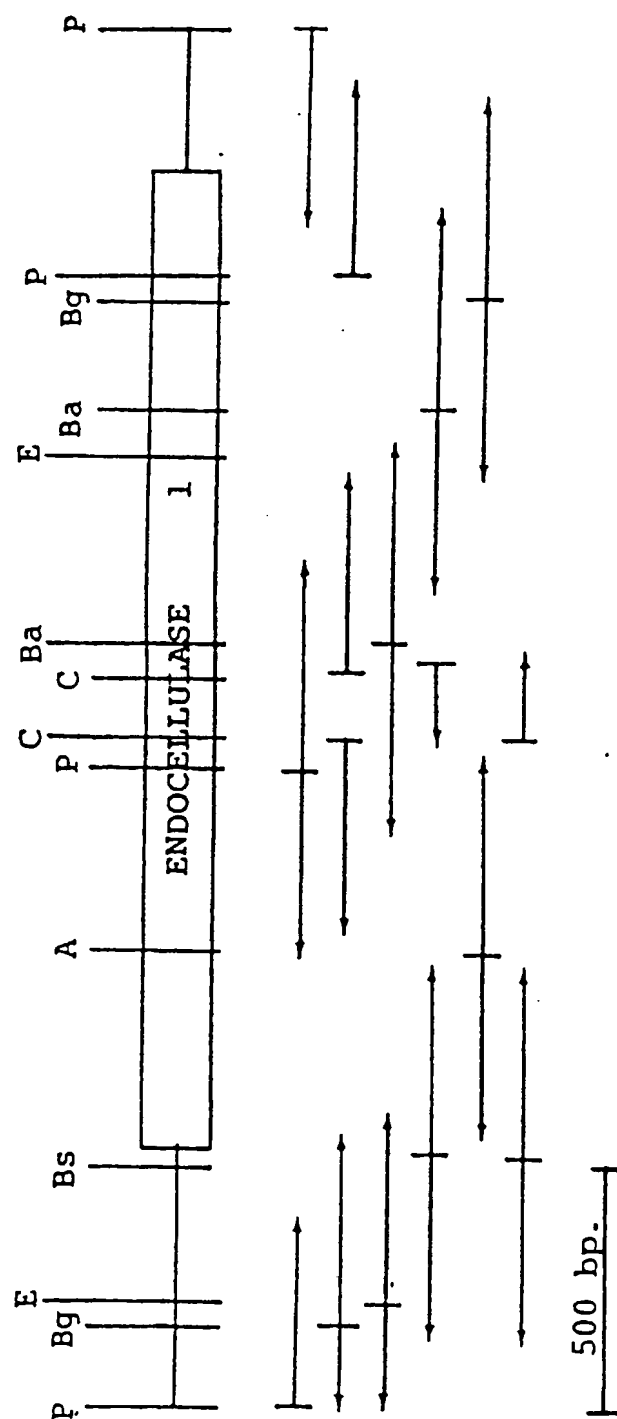


Fig. 4

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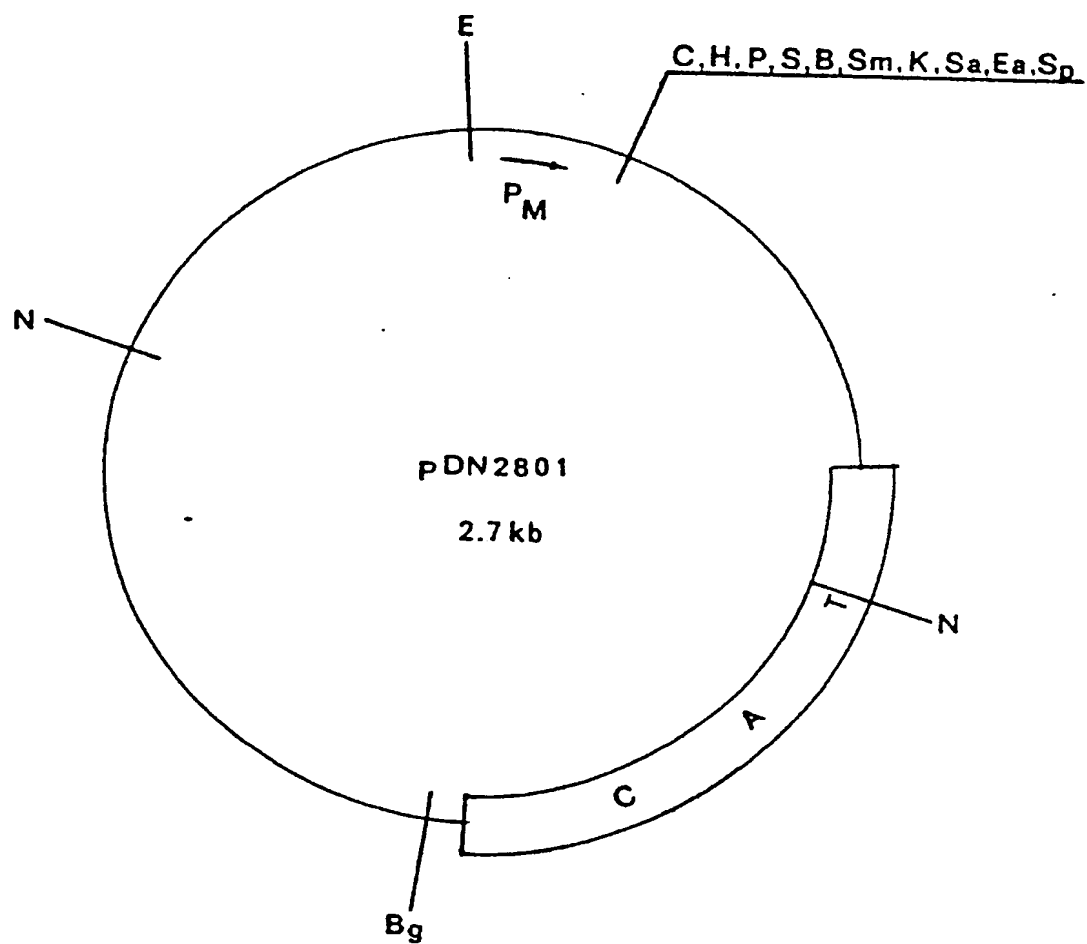


Fig. 5

REPLACEMENT SHEET

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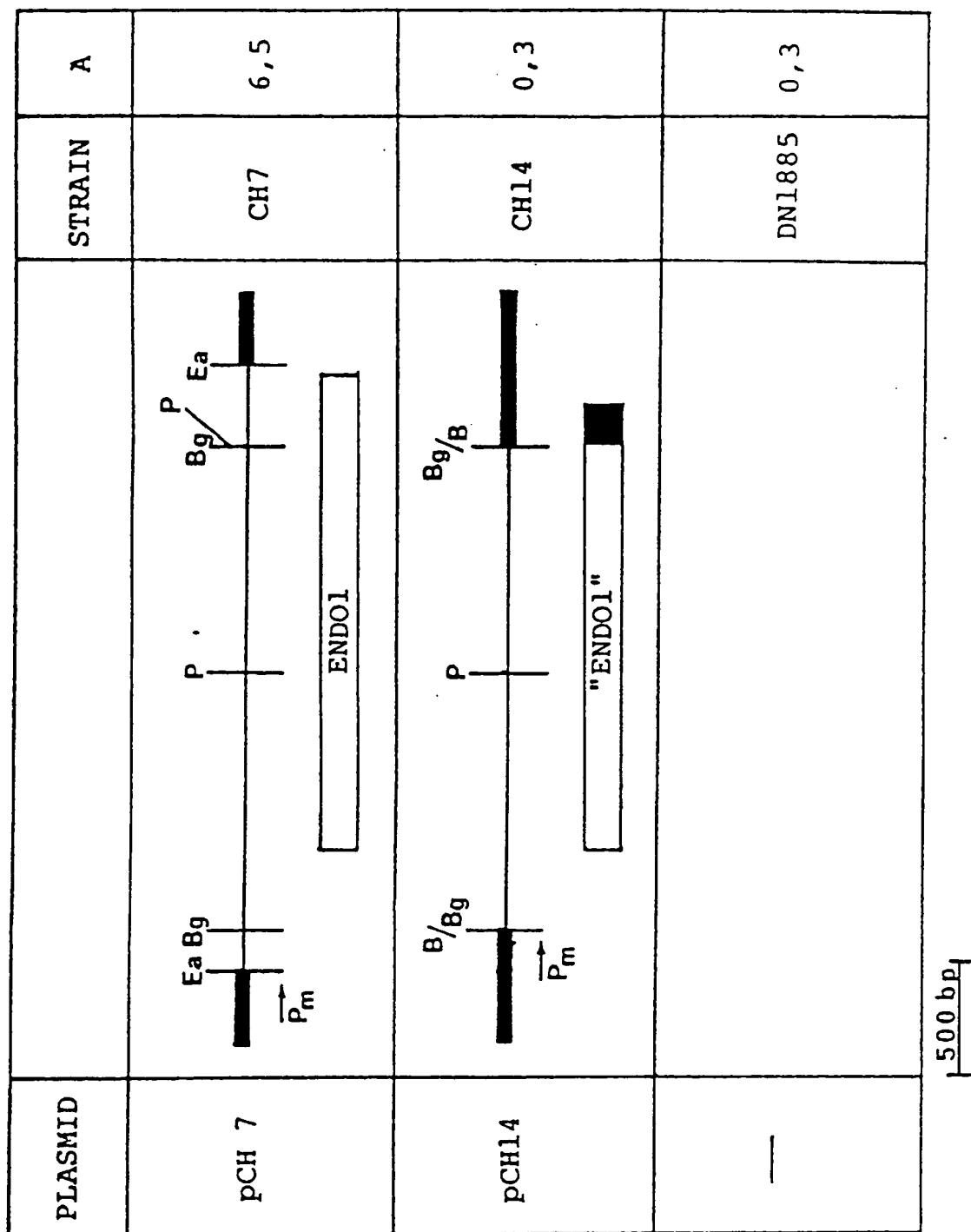


Fig. 6

REPLACEMENT SHEET



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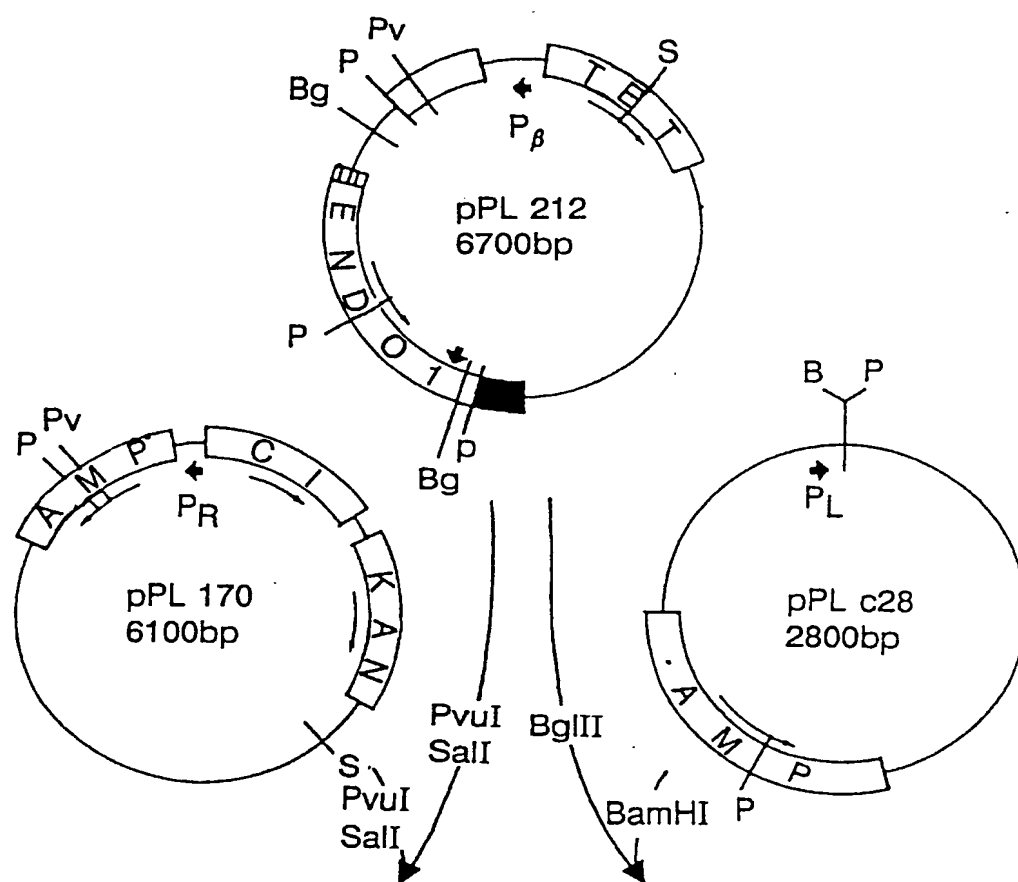


Fig. 7a

REPLACEMENT SHEET

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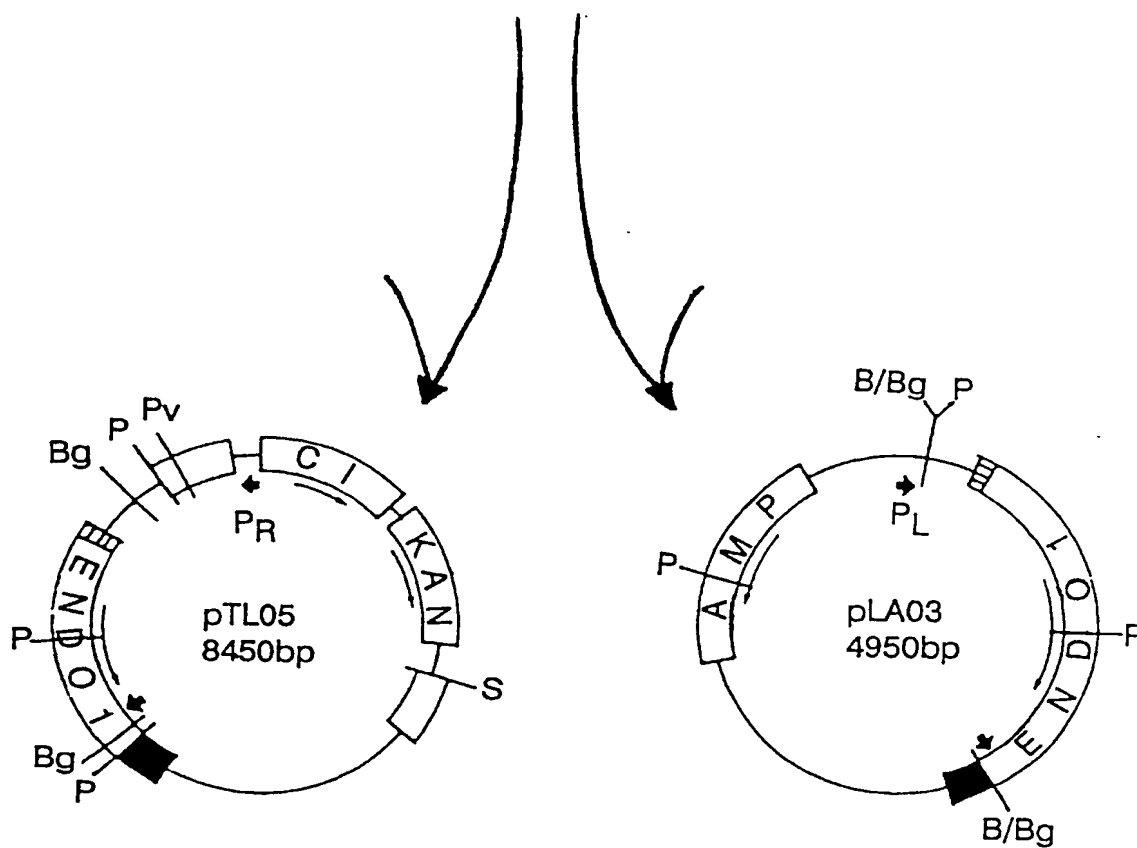


Fig. 7b

GEN. ACQUISITION

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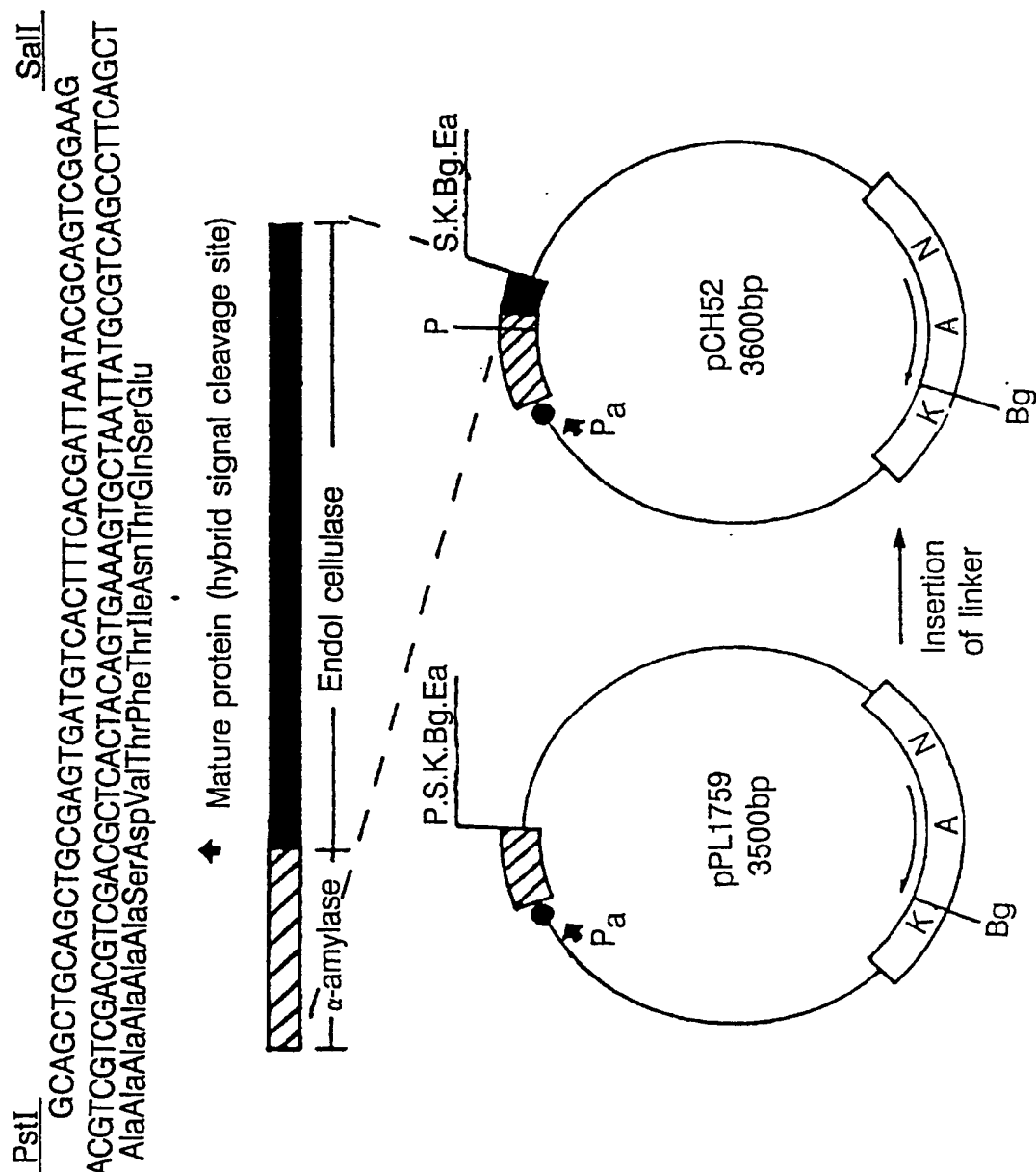
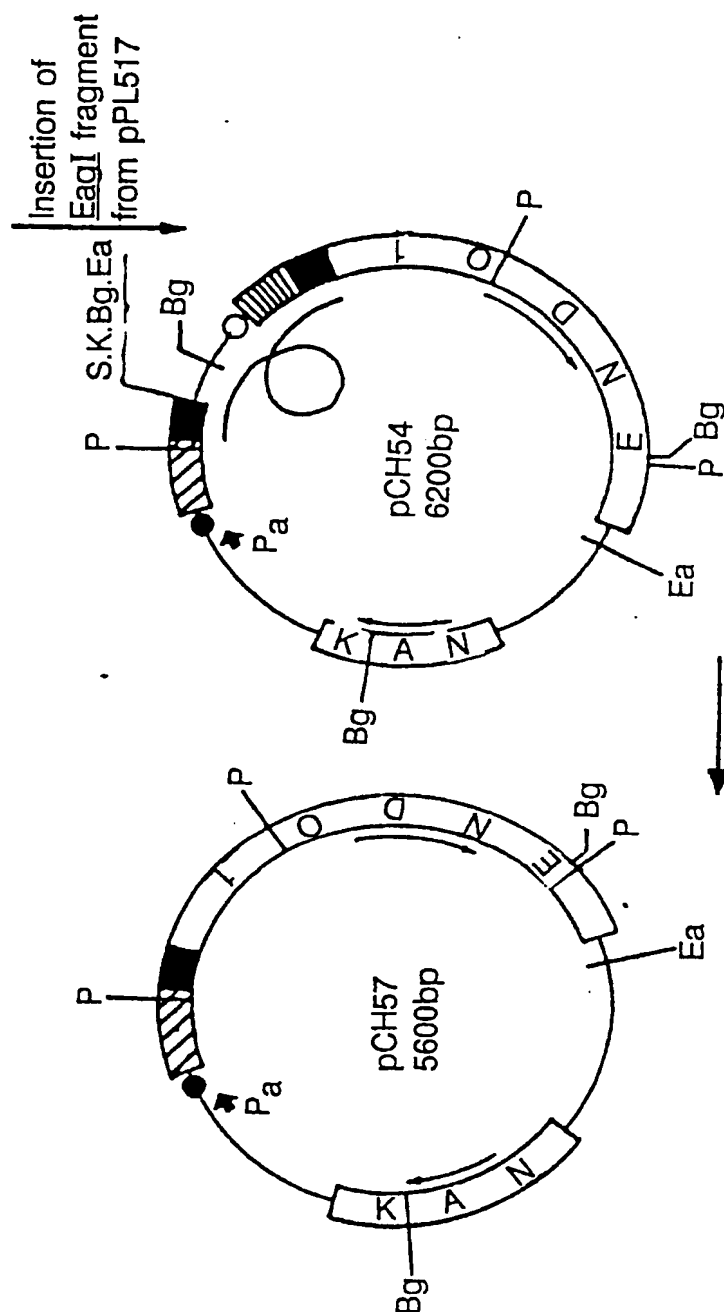


Fig. 8a

REPLACEMENT SHEET

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- 1: In vivo recombination
- 2: Enrichment cutting with *KpnI*
- 3: Transformation

Fig. 8b

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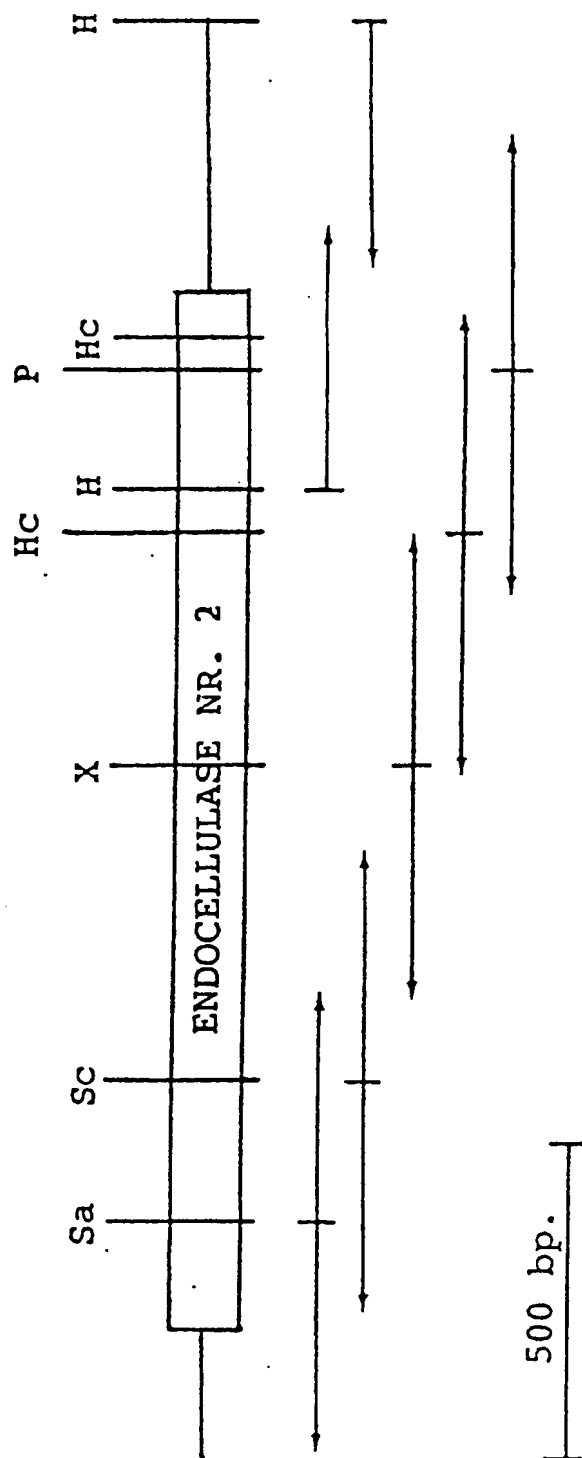


Fig. 9

REPLACEMENT SHEET

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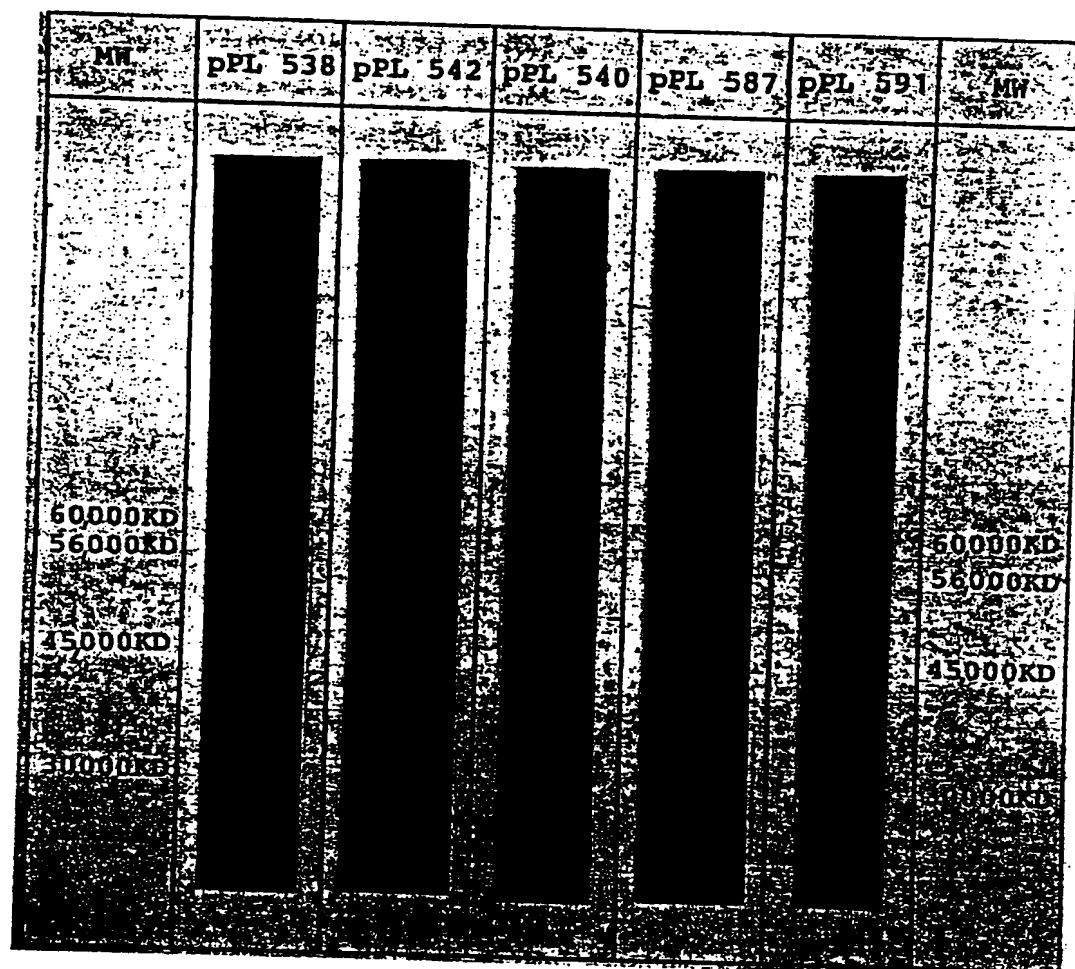


Fig. 10

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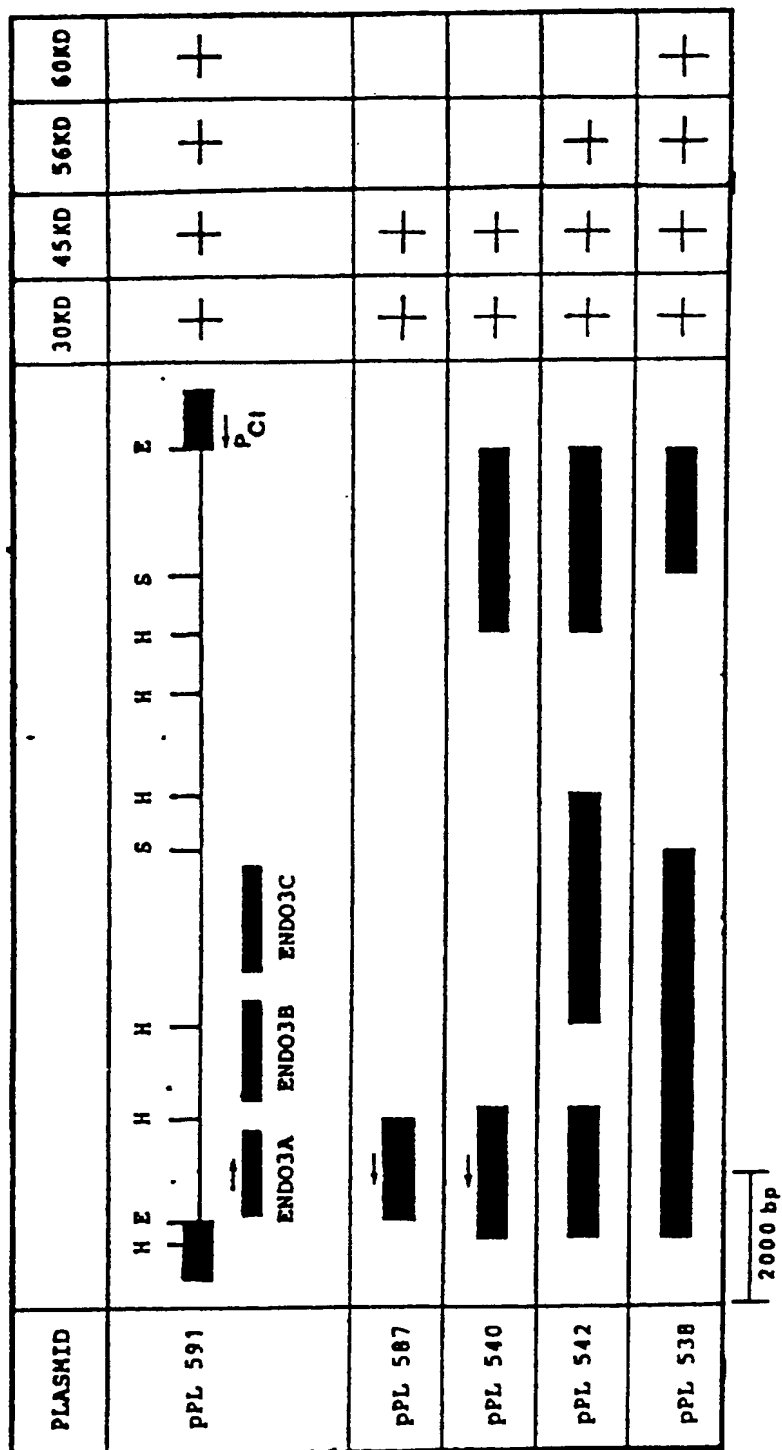


Fig. 11

REPLACEMENT SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00013

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 9/42, C 12 N 15/56, C 11 D 3/386																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">IPC5</td> <td style="height: 40px; vertical-align: bottom;">C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched<sup>8</sup></div>			Classification System	Classification Symbols	IPC5	C 12 N											
Classification System	Classification Symbols																
IPC5	C 12 N																
SE,DK,FI,NO classes as above																	
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category<sup>*</sup></th> <th style="width: 60%;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>EP, A2, 0270974 (KAO CORPORATION) 15 June 1988, see example 7, pages 44-47 --</td> <td style="text-align: center; vertical-align: top;">1-11,14-42</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>EP, A2, 0269977 (KAO CORPORATION) 8 June 1988, see the claims --</td> <td style="text-align: center; vertical-align: top;">2-11,15-42</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>EP, A2, 0271004 (KAO CORPORATION) 15 June 1988, see the whole document --</td> <td style="text-align: center; vertical-align: top;">1-11,14-42</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>EP, A2, 0265832 (KAO CORPORATION) 4 May 1988, see the whole document --</td> <td style="text-align: center; vertical-align: top;">1-11,14-42</td> </tr> </tbody> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	EP, A2, 0270974 (KAO CORPORATION) 15 June 1988, see example 7, pages 44-47 --	1-11,14-42	X	EP, A2, 0269977 (KAO CORPORATION) 8 June 1988, see the claims --	2-11,15-42	A	EP, A2, 0271004 (KAO CORPORATION) 15 June 1988, see the whole document --	1-11,14-42	A	EP, A2, 0265832 (KAO CORPORATION) 4 May 1988, see the whole document --	1-11,14-42
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>															
X	EP, A2, 0270974 (KAO CORPORATION) 15 June 1988, see example 7, pages 44-47 --	1-11,14-42															
X	EP, A2, 0269977 (KAO CORPORATION) 8 June 1988, see the claims --	2-11,15-42															
A	EP, A2, 0271004 (KAO CORPORATION) 15 June 1988, see the whole document --	1-11,14-42															
A	EP, A2, 0265832 (KAO CORPORATION) 4 May 1988, see the whole document --	1-11,14-42															
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p><sup>"A"</sup> document defining the general state of the art which is not considered to be of particular relevance</p> <p><sup>"E"</sup> earlier document but published on or after the international filing date</p> <p><sup>"L"</sup> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p><sup>"O"</sup> document referring to an oral disclosure, use, exhibition or other means</p> <p><sup>"P"</sup> document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p><sup>"T"</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p><sup>"X"</sup> document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p><sup>"Y"</sup> document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p><sup>"&amp;"</sup> document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="height: 30px; vertical-align: bottom;">6th May 1991</td> <td style="height: 30px; vertical-align: bottom; text-align: center;">1991-05-13</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;">Signature of Authorized Officer</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom; text-align: center;">SWEDISH PATENT OFFICE</td> <td style="height: 40px; vertical-align: bottom; text-align: center;">            Yvonne Siösteen         </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	6th May 1991	1991-05-13	International Searching Authority	Signature of Authorized Officer	SWEDISH PATENT OFFICE	Yvonne Siösteen							
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report																
6th May 1991	1991-05-13																
International Searching Authority	Signature of Authorized Officer																
SWEDISH PATENT OFFICE	Yvonne Siösteen																



III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 8909259 (NOVO INDUSTRI A/S) 5 October 1989, see page 4, line 7 - line 9 --	1-11,14- 42
Y	Chemical Abstracts, volume 108, no. 19, 9 May 1988, (Columbus, Ohio, US), Warren, R.A.J et al: "A bifunctional exoglucanase-endoglucanase fusion protein ", see page 295, abstract 163739k, & Gene 1987, 61( 3), 421- 427 ---	12,13
Y	Chemical Abstracts, volume 110, no. 23, 5 June 1989, (Columbus, Ohio, US), Greenwood, Jeffrey M et al: "Fusion to an endoglucanase allows alkaline phosphatase to bind to cellulose ", see, abstract 208834x, & FEBS Lett. 1989, 244( 1), 127- 131 --	12,13
Y	Chemical Abstracts, volume 111, no. 19, 6 November 1989, (Columbus, Ohio, US), Kilburn, D.G. et al: "Cellulases of Cellulomonas fimi. The enzymes and their interactions with substrate ", see page 331, abstract 170011g, & ACS Symp.Ser. 1989, 399(), 587- 596 --	12,13
A	Chemical Abstracts, volume 111, no. 21, 20 November 1989, (Columbus, Ohio, US), Ong, Edgar et al: "The cellulose-binding domains of cellulases: tools for biotechnology ", see page 619, abstract 192974a, & Trends Biotechnol. 1989, 7( 9), 239- 243 -- -----	12,13

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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/partly/

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claim numbers 1, 14, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
 The wording of these claims are too broadly formulated to permit a meaningful search of the whole claims.
  
3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00013**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 91-03-23. The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0270974	88-06-15	JP-A- 63273474	88-11-10
		US-A- 4943532	90-07-24
		JP-A- 63273475	88-11-10
		JP-A- 63279790	88-11-15
		JP-A- 1037286	89-02-07
		JP-A- 1112982	89-05-01
		JP-A- 63141586	88-06-14
EP-A2- 0269977	88-06-08	US-A- 4962030	90-10-09
		JP-A- 1037285	89-02-07
		JP-A- 1037287	89-02-07
		JP-A- 1037288	89-02-07
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		JP-A- 63240777	88-10-06
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		US-A- 4978470	90-12-18
EP-A2- 0265832	88-05-04	JP-A- 63109771	88-05-14
		US-A- 4945053	90-07-31
		JP-A- 63109776	88-05-14
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WO-A1- 8909259	89-10-05	EP-A- 0406314	91-01-09

